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Epigenetic regulation of flowering in *Arabidopsis thaliana*

Kathryn Anne Kovac

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Statement of Originality

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University, To the best of my knowledge, this thesis contains no material previously published, or the result of any work by any other person, except where due reference is made in the text.

Kathryn Anne Kovac

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This thesis is dedicated to the memory of my Nana,

Nancy Caroline Westerman

July 1909 – May 1997

from your Katie

Abstract

The reversible methylation of cytosine residues in DNA makes an important contribution to the epigenetic regulation of gene expression in many organisms. In plants, perturbation of DNA methylation causes a wide spectrum of phenotypic abnormalities, including an alteration of flowering time.

There are a number of DNA methyltransferases in *Arabidopsis thaliana*. *Arabidopsis* plants were transformed with silencing constructs against the methyltransferase *METII*. These plants did not show a significant decrease in global, CG or CNG methylation levels and had no morphological phenotypic abnormalities, yet they flowered significantly earlier than wild type plants. The extent of the promotion of flowering in *METII* transgenic lines correlated with the level of reduction in *METII* expression. Unlike *METI* antisense plants, in which flowering is promoted by a decrease in expression of the MADS-box transcription factor *FLC, METII* transgenic plants did not have any detectable change in *FLC* expression. Instead, early flowering in a *METII* transgenic line was correlated with up-regulation of several genes involved in photosynthesis. Together, these results suggested that METII methylates highly specific sequences and that it may regulate the expression of either photosynthetic genes or an upstream regulator of these genes.

Demethylation of DNA in the *Arabidopsis* C24 ecotype caused by an antisense construct against the *METI* methyltransferase also promotes flowering. However, demethylation of DNA caused by mutation of the *DECREASE IN DNA METHYLATION* (*DDM1*) gene in the Columbia ecotype delays flowering. Backcrossing the anti-methyltransferase (AMT) line

and *ddm1* mutant into Landsberg *erecta* lines containing dominant alleles of the floral repressor *FLC* showed that like AMT, *ddm1* was also able to down-regulate *FLC* expression. As the single-copy gene *FWA* was not demethylated in the *ddm1* plants, this suggests that down-regulation of *FLC* expression is likely to involve repeat sequence demethylation and may occur via an effect on chromatin structure, rather than as a result of single copy sequence demethylation.

Interactions between vernalisation, gibberellic acid (GA) and demethylation of DNA in the promotion of flowering in the C24 ecotype were investigated by crossing the GA non-responsive mutant *gai* to *METI* antisense plants. GA non-responsive F1 plants responded to vernalisation, consistent with GA and vernalisation promoting flowering via separate pathways. Demethylation mainly promoted flowering via an *FLC*-dependent, GA-independent pathway. However, *gai* partly blocked the demethylation-induced promotion of flowering, suggesting that demethylation might also promote flowering via a GA-dependent pathway.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

AMVRT	avian myeloblastosis virus reverse transcriptase
ATP	adenosine triphosphate
BSA	bovine serum albumen
CAPS	cleaved amplified polymorphic sequence
cDNA	complementary DNA
CsCl	caesium chloride
CTAB	cetyltrimethyl ammonium bromide
CTP	cytosine triphosphate
d	day
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
DEPC	diethyl pyrocarbonate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
dNTP	deoxynucleotide triphosphate
dTTP	deoxythymine triphosphate
EDTA	[ethylenedinitrilo]-tetraacetic acid disodium salt
EtBr	ethidium bromide
GA	gibberellic acid
GTP	guanosine triphosphate
h	hour
KCl	potassium chloride
LB	Luria-Bertoni
MES	2-[N-morpholino]ethanesulfonic acid
min	minute
MOPS	3-[N-morpholino]propanesulfonic acid
MS	Murashige-Skoog
NPTII	neomycinphosphotransferase II
NaCl	sodium chloride
PCR	polymerase chain reaction
RNA	ribonucleic acid
RNAse	ribonuclease
mRNA	messenger RNA
RP-HPLC	reversed-phased high performance liquid chromatography
RT	room temperature; reverse transcriptase
RT-PCR	reverse transcriptase-polymerase chain reaction
SDS	sodium dodecylsulphate
sec	second
Sf-2	San Feliu-2
SSC	salt sodium chloride
TAE	Tris acetate EDTA
TE	Tris-EDTA
Tris	Tris(hydroxymethyl)aminomethane
Triton-X 100	t-octylphenoxypolyethoxyethanol
TsAP	thermosensitive alkaline phosphatase
TTP	thymine triphosphate
vol	volumes

Chapter 1: General Introduction

1.1 DNA methylation

Methylation of cytosine residues within a DNA sequence enables an increase in the amount of information held by that sequence without altering the base composition. DNA methylation has been implicated as playing a role in a diverse array of functions including regulation of gene expression, gene silencing, imprinting, X-chromosome inactivation and genome defence, in bacteria, plants and animals. In humans, abnormalities in DNA methylation have been implicated in cancer, immunodeficiency diseases and mental retardation, demonstrating the importance of DNA methylation in development (reviewed in Jones and Takai, 2001).

DNA methylation is one of several phenomena broadly grouped under the banner of "epigenetics", a concept which defines a heritable but potentially reversible change in gene expression (reviewed in Lewin, 1998). Historically, DNA methylation has usually been correlated with a reduction in gene expression (reviewed in Jost and Saluz, 1993). In the past decade, this simple correlation has been greatly extended and DNA methylation has been identified as one of many players involved in a complex system of interacting factors that cause alterations to the higher order structure of chromatin and concomitant changes in levels of gene transcription. However, the precise nature of the contribution of DNA methylation to the molecular mechanisms of transcriptional regulation is yet to be completely elucidated and is currently the object of intensive study.

1.2 Frequency and sites of DNA methylation

Levels and sites of DNA methylation vary enormously among different organisms. In mammals, about 2-7 % of cytosines are methylated (Razin and Riggs, 1980), with most of this methylation occurring within CG dinucleotides (Bird, 1986). In general, plants have much higher levels of DNA methylation than mammals. The level of methylation depends on the species, ranging from 5-6 % mC in *Arabidopsis thaliana* (Leutwiler *et al.,* 1984; Kakutani *et al.,* 1999) to 33 % in rye (Thomas and Sherratt, 1956). Methylation of plant DNA occurs within many different sequence contexts; the majority occurs at CG sites, but CNG (where N is any other nucleotide) and asymmetric (non CG or CNG) methylation also occurs (Bender and Fink, 1995; Gruenbaum *et al.,* 1981; Jeddeloh and Richards, 1996; Meyer *et al.,* 1994; Oakley and Jost, 1996). Sites of methylation are not random, with a high frequency of methylation found within repetitive DNA sequences (Rabinowicz *et al.,* 1999).

1.3 Inheritance of methylation patterns

The process of DNA methylation involves the transfer of a methyl group from the co-factor S-adenosyl-L-methionine (AdoMet) to the C5 position of cytosine, a reaction which is catalysed by methyltransferase enzymes. Patterns of methylation within symmetrical sites such as CG are inherited by a process termed maintenance methylation. The DNA molecule is methylated on both strands, but after replication only the cytosines on the parental DNA strand contain methyl groups, while the newly replicated DNA strand is unmodified. The transfer of methyl groups occurs immediately after DNA replication, when the loosened chromatin structure allows for access by methyltransferase enzymes (Adams, 1995). The hemi-methylated DNA acts as a template for methylation of the

corresponding cytosine residue on the nascent strand of DNA (Bird, 1978; Holliday and Pugh, 1975). The so-called "maintenance" methyltransferases have a distinct preference for such hemi-methylated substrates (Bestor and Ingram, 1983).

DNA methyltransferases can also introduce patterns of methylation *de novo*, without a pre-existing hemi-methylated template (Adams and Lindsay, 1993; Bestor and Ingram, 1983), resulting in asymmetric methylation patterns (Bender and Fink, 1995; Meyer *et al.*, 1994; Oakely and Jost, 1996). Such patterns are thought to be perpetuated by *de novo* methyltransferase activity that is distinct from maintenance activity (Wassenegger and Pelissier, 1998). As *de novo* methylation does not rely on a template, there must be a different type of signal for asymmetric methylation to be initiated. Structural elements such as secondary DNA structures or chromatin conformation were initially suggested as candidates for such a signal (Meyer *et al.*, 1994), but more recently, an RNA-directed guiding system has been implicated in the targeting of methyltransferases to specific sequences (Wassenegger *et al.*, 1994; Pelissier *et al.*, 1999).

De novo methylation is implicated in the spreading of methylation along DNA from a focal point, and was first noted to occur on sequences flanking the integration site of provirus DNA in mice germ line cells (Jahner and Jaenisch, 1985). Methylation spreading could be catalysed by the same enzyme that performs maintenance methylation, or by a separate enzyme/s. The murine and human maintenance methyltransferases have a preference for partially methylated DNA over unmethylated DNA (Carotti *et al.*, 1998), and a methyl-DNA binding domain of the murine Dnmt1 enzyme has recently been shown to allosterically activate its catalytic region (Fatemi *et al.*, 2001). This suggests that the presence of a small number of methylated cytosines could induce further methyltransferase activity, and hence spreading of methylation.

1.4 DNA methyltransferase enzymes

1.4.1 Prokaryotic methyltransferases

In bacteria, methylation acts as part of the well-characterised restriction/modification system of genome defence. In this system, foreign DNA is cleaved by site-specific endonucleases, but self DNA containing the same target sequence is methylated and protected by the cognate methyltransferases (Noyer-Weidner and Trautner, 1993).

Prokaryotic methyltransferases consist of one domain containing ten conserved amino acid motifs (Posfai *et al.*, 1989), six of which are highly conserved (Kumar *et al.*, 1994). The amino acid motifs are usually found in a defined order of I-X (Posfai *et al.*, 1989), but enzymes with different arrangements of motifs have also been identified (Xu *et al.*, 1997; Jeltsch, 1999). The conserved sequence motifs constitute the core of the three-dimensional structure of the enzyme and are involved in cofactor binding and enzyme function (Cheng *et al.*, 1993). The cofactor AdoMet is bound by motifs I and X (Ingrosso *et al.*, 1989) and motif IV contains the enzyme's active site (Wu and Santi, 1987) into which the target cytosine is extruded from the DNA helix (Klimasauskas *et al.*, 1994; Kumar *et al.*, 1994). Motif IV contains an absolutely conserved proline-cysteine doublet which is necessary for the methyl transfer reaction (Chen *et al.*, 1991; Wyszynski *et al.*, 1991). Studies of the M. *Hha I* methyltransferase suggest that as well as binding the flipped-out base, the enzyme plays an active role in opening the DNA duplex (Klimasauskas *et al.*, 1998). A variable region, termed the target recognition domain (TRD), lies between motifs VIII and IX. The TRD defines the enzyme's sequence specificity and is involved in

directing the methyltransferase to the target cytosine (Balganesh *et al.*, 1987; Trautner *et al.*, 1988; Wilke *et al.*, 1988; Klimasauskas *et al.*, 1991).

1.4.2 Mammalian methyltransferases

Eukaryotic methyltransferases are generally more complex than their prokaryotic counterparts, and can be grouped into different classes. Enzymes such as those belonging to the Dnmt1 class of mammalian methyltransferases have two distinct domains. They are thought to have evolved from ancestral prokaryotic-like enzymes via the fusion of a gene encoding an unrelated DNA-binding protein to the ancestral methyltransferase gene, resulting in a protein containing two domains; an amino-terminal domain and a catalytic carboxy-terminal domain (Bestor, 1990). The C-terminal methyltransferase domain contains 8 of the 10 conserved amino acid motifs identified in prokaryote enzymes (Bestor *et al.*, 1988). The N-terminal domain interacts with a variety of proteins involved in chromatin organization, structure and gene regulation (Fatemi *et al.*, 2001). It contains a zinc-binding motif that is involved in the discrimination of hemi-methylated and unmethylated DNA (Bestor, 1992); this motif also interacts with the catalytic domain, causing enzyme activation (Fatemi *et al.*, 2001).

Targeting sequences in the N-terminal domain direct the enzyme to the replication fork during the S-phase of the cell cycle (Leonhardt *et al.*, 1992). These sequences include a bromo-adjacent (BAH) domain similar to those found in several proteins involved in regulation of transcription (Liu *et al.*, 1998; Callebaut *et al.*, 1999). Further evidence of the localisation of methyltransferases to newly replicated DNA comes from the identification of interactions between methyltransferases and proliferating-cell nuclear antigen (PCNA) (Chuang *et al.*, 1997). PCNA is an auxiliary factor for DNA replication and repair that

encircles and slides along DNA. Its association with methyltransferases may mediate their localisation (reviewed in Kelman and Hurwitz, 1998).

The *Dnmt1* gene of mice was the first mammalian methyltransferase gene to be isolated (Bestor *et al.*, 1988); a homologue has also been identified in humans (DNMT1; Yen *et al.*, 1992). *Dnmt1* knock-out mouse embryos containing 30 % of wild type methylation levels are stunted and abort midway through the gestation period (Li *et al.*, 1992), demonstrating the importance of DNA methylation in development. Dnmt1 preferentially methylates CG sequences (Yoder *et al.*, 1997) and is generally thought to act as a maintenance methyltransferase, but it has also been shown to have *de novo* methylating activity *in vitro* (Yoder *et al.*, 1997) and *in vivo* in *E. coli* cells (Tollefsbol and Hutchison, 1998).

A second class of mammalian methyltransferase, Dnmt2, contains all the conserved amino acid motifs of other methyltransferases (Okano *et al.*, 1998a) but lacks an amino-terminal regulatory domain (Yoder and Bestor, 1998), so is more similar to prokaryotic methyltransferases than it is to Dnmt1. Dnmt2 is not required for either *de novo* or maintenance methylation in embryonic stem cells (Okano *et al.*, 1998a) and its biological function is as yet unknown. The human DNMT2 is able to bind DNA via a specific recognition motif, but has perhaps lost the ability to transfer methyl groups (Dong *et al.*, 2001). A homologue of Dnmt2 has also been identified in *Schizosaccharomyces pombe* (Wilkinson *et al.*, 1995), an organism which lacks detectable DNA methylation. The *S. pombe* Dnmt2 homologue has a serine separating the conserved proline-cysteine doublet (Wilkinson *et al.*, 1995). Deletion of the serine restores methyltransferase activity (Pinabarsi *et al.*, 1996), suggesting that as for the human DNMT2, the *S. pombe* homologue has lost the ability to methylate DNA.

The third class of mammalian methyltransferase has two members, Dnmt3a and Dnmt3b (Okano *et al.,* 1998b). The existence of these *de novo* methyltransferases was predicted by the methylation of proviral DNA occurring at the same rate in both wild type cells and cells lacking Dnmt1 activity (Lei *et al.,* 1996). In mice, Dnmt3a and Dnmt3b are required for the genome-wide *de novo* methylation that occurs after blastocyst implantation (Okano *et al.,* 1999; Ramsahoye *et al.,* 2000) and are partly redundant in their function, as double mutants are embryo lethal, but single mutants are not (Okano *et al.,* 1999). However, they do not have exactly the same function, as single mutants of either enzyme show different developmental defects, and minor satellite repeats are demethylated in *Dnmt3b* mutants but not in *Dnmt3a* mutants (Okano *et al.,* 1999). The human DNMT3a and 3b are 94-98 % identical to the mouse enzymes (Xie *et al.,* 1999). DNMT3b methylates specific centromeric repeats and is thought to be responsible for stabilisation and organisation of satellite DNA, with mutations in DNMT3b causing the human immunodeficiency syndrome ICF (Xu *et al.,* 1999). DNMT3b has recently been shown to act cooperatively with DNMT1 to maintain methylation and silencing in human cancer cells (Rhee *et al.,* 2002).

1.4.3 Plant methyltransferases

Plant methyltransferases were first identified in wheat (Theiss *et al.,* 1987) and methyltransferase enzymes have been cloned from *Arabidopsis* (Finnegan and Dennis, 1993; Henikoff and Comai, 1998; Genger *et al.,* 1999; Cao *et al.,* 2000), pea (Pradhan and Adams, 1995; Pradhan *et al.,* 1998), carrot (Bernacchia *et al.,* 1998a), tomato (Bernacchia *et al.,* 1998b), tobacco (Nakano *et al.,* 2000) and maize (Papa *et al.,* 2001).

Arabidopsis is thought to contain at least ten methyltransferase genes (The *Arabidopsis* Genome Initiative, 2000), which fall into at least three classes. The *METI* class of

methyltransferases is most similar to the mammalian *Dnmt1* class (Finnegan and Dennis, 1993), although there are two major differences between them. The N-terminal domain of METI lacks the cysteine-rich zinc-binding motif of the mammalian enzyme (Bestor, 1992) but contains an acidic region which is conserved among plant methyltransferases (Genger *et al.,* 1999). *METI* is 50 % identical to *Dnmt1* in the methyltransferase domain, with identity falling to 24 % in the N-terminal domain (Finnegan and Dennis, 1993). Like *Dnmt1, METI* is thought to preferentially methylate CG dinucleotides (Finnegan *et al.,* 1996; Kishimoto *et al.,* 2001). Other members of the *METI* class are *METIIa, METIIb*, and *METIII* (Genger *et al.,* 1999). *METIIa* is 68-79 % identical to *METI*, and *METIIb* is over 90 % identical to *METIIa*. No roles have yet been assigned to these enzymes. The third gene, *METIII*, is non-essential and encodes a truncated protein in one ecotype of *Arabidopsis* (Genger *et al.,* 1999) but not in all of them (EJ Finnegan, personal communication).

Evidence for the existence of more than one class of methyltransferases in plants came from experiments showing that CNG methylation and regions of hypermethylation occurred in plants in which METI activity had been reduced (Finnegan *et al.,* 1996; Ronemus *et al.,* 1996; Jacobsen and Meyerowitz, 1997). The second class of methyltransferase identified, the chromomethyltransferase (*CMT*) class, has so far been found only in plants (Henikoff and Comai, 1998; Rose *et al.,* 1998). Although the CMT class of enzymes shares homology with other methyltransferases, CMTs are distinguished by the presence of a chromodomain within the catalytic domain. The chromodomain is thought to be involved in mediating interactions between chromatin components and guiding CMTs to regions of heterochromatin (Henikoff and Comai, 1998). There are three known members of the *CMT* class. *CMT1,* which lacks the long N-terminal domain of other methyltransferases, is predicted to be non-functional (Henikoff and Comai, 1998). *CMT2* is 60-80 % identical to *CMT1* in the methyltransferase domain, but it has a long amino-

terminal domain which bears little resemblance to those of the *METI* class (Genger *et al.*, 1999). The third member of the *CMT* class, *CMT3*, has been ascribed roles in both CG and CNG methylation (Bartee *et al.*, 2001; Lindroth *et al.*, 2001). *CMT3* preferentially methylates CNG sites within transposons (Tompa *et al.*, 2002) and is targeted to heterochromatin via an interaction with a homologue of heterochromatin protein 1 (Jackson *et al.*, 2002). A maize gene, *Zmet2*, which has similarities to both *CMT1* and *CMT3*, shows specificity only for CNG methylation (Papa *et al.*, 2001).

The third class of plant methyltransferase enzymes are known as domains rearranged methyltransferases (DRMs). As the name suggests, these enzymes have a novel arrangement of motifs, with motifs VI, IX and X found before motifs I-IV (Cao *et al.*, 2000). Enzymes with non-canonical arrangements of motifs also exist in some bacteria (Jeltsch, 1999). DRM's, which have so far been identified in maize (*ZMet3*) and *Arabidopsis* (*DRM1* and *DRM2*), have a high similarity to the *Dnmt3* family, suggesting that they function as *de novo* methyltransferases (Cao *et al.*, 2000). DRM's also contain a ubiquitin-associated (UBA) domain, reminiscent of those found in ubiquitin pathway enzymes. The function of the UBA domain in the DRM class of enzyme is not yet clear (Cao *et al.*, 2000).

Another plant methyltransferase gene has been identified (accession #AF045889) that is homologous to the mammalian Dnmt2 enzyme (Finnegan and Kovac, 2000). As yet no function has been described for this gene, which could represent a fourth class of plant methyltransferases.

1.4.4 Other methyltransferases

Methyltransferase enzymes have been identified in many different animal species (Tajima *et al.,* 1995; Aniello *et al.,* 1996; Kimura *et al.,* 1996; Xie *et al.,* 1999), as well as in many non-animal species. The fungus *Ascobolus immersus* has two methyltransferase genes, *MASC1* and *MASC2*. MASC1, like Dnmt2, lacks the regulatory N-terminal domain (Malagnac *et al.,* 1997). MASC1 has no detectable *in vitro* activity, and disrupting it does not affect maintenance methylation, but does affect the methylation induced pre-meiotically (MIP) gene silencing process (Malagnac *et al.,* 1997). Unlike MASC1, MASC2 has an N-terminal domain and *in vitro* methyltransferase activity, but disrupting its expression has no effect (Malagnac *et al.,* 1999), suggesting the existence of a third methyltransferase in this species. In contrast to *Ascobolus,* disruption of the *Neurospora Dim-2* methyltransferase gene, which has a novel N-terminal domain unlike those of any other methyltransferase, eliminates all detectable DNA methylation within both symmetric and asymmetric sequences. This indicates that Dim-2 might be capable of both maintenance and *de novo* activity, and hence might be the only methyltransferase in Neurospora (Kouzminova and Selker, 2001).

The fruitfly *Drosophila melanogaster,* originally thought to lack DNA methylation (Urieli-Shoval *et al.,* 1982), is now known to contain very low levels that are detectable only during early embryo development (Gowher *et al.,* 2000; Lyko *et al.,* 2000). *Drosophila* has two methyltransferase proteins, DmMTR1 and DmMTR2, that are related to Dnmt1 and Dnmt2 respectively (Hung *et al.,* 1999). Identification of non-CG methylation in *Drosophila* embryos has prompted speculation that the mammalian Dnmt2 enzyme might methylate these sequences (Lyko, 2001).

1.5 Roles and functions of DNA methylation

1.5.1 Methylation and chromatin

DNA methylation has been attributed roles in many developmental and regulatory processes, underlying all of which is the regulation of gene expression. In this role, DNA methylation does not act as a solitary player, but contributes to the control of both activation and suppression of transcription in combination with many other factors, including histone proteins, proteins that bind methylated DNA and macromolecular complexes known as chromatin remodelling machines.

The DNA duplex is tightly wrapped around histone proteins, the amino-terminal "tails" of which can be subjected to a variety of modifications, including acetylation, methylation, phosphorylation and ubiquitination. Like DNA methylation, the combination of histone modifications adds a further level of epigenetic complexity to the information stored within the genetic code (Strahl and Allis, 2000). The acetylation status of the histone tails influences their interaction with DNA. Acetylation of histone tails, carried out by histone acetyltransferases (HATs), is associated with an open, active conformation, whereas deacetylation, caused by the action of histone deacetyltransferases (HDACs) is associated with an inactive, repressive conformation (reviewed in Knoepfler and Eisenmann, 1999). HATs and HDACs are components of chromatin remodelling complexes, ATP-dependent machines that alter histone-DNA interactions within nucleosomes. These alterations remodel the chromatin between "on" and "off" states, by either allowing or denying access to factors required for transcription (Peterson and Tamkun, 1995; Sudarsanam and Winston, 2000).

The identification of proteins that could bind to methylated DNA, such as MeCP1 (Meehan *et al.*, 1989) and MeCP2 (Lewis *et al.*, 1992) and the realisation that they interact with co-repressor complexes that in turn interact with HDACs, indicated that DNA methylation can lead to chromatin modification (Hendrich and Bird, 1998; Jones *et al.*, 1998; Nan *et al.*, 1998; Ng *et al.*, 1999). This discovery led to the proposal that methylated DNA might serve as a signal for the inheritance of stable repressive chromatin states via recruitment of methyl-binding proteins (Eden *et al.*, 1998; Ng and Bird, 1999). Reducing HDAC activity reactivates some normally silenced genes without affecting methylation levels (Tian and Chen, 2001), suggesting that methylation could recruit HDACs, or act upstream of histone deacetylation. However, it is unlikely that histone modification could be totally dependent on DNA methylation, as transcription is also regulated by HDAC activity in DNA methylation-deficient species such as yeast (reviewed in Struhl, 1998). Selective loss of DNA methylation and reactivation of certain genes in *Neurospora* after treatment with the HDAC inhibitor Trichostatin A initially suggested that the acetylation state of histones could control DNA methylation (Selker, 1998), and in *Neurospora* and *Arabidopsis*, DNA methylation has been shown to be dependent on histone methylation (Tamaru and Selker, 2001; Jackson *et al.*, 2002). However, it has also recently been shown that a decrease in DNA methylation leads to a decrease in histone H3 methylation, suggesting that methylated DNA can direct methylation of histones (Soppe *et al.*, 2002). Both these observations can be reconciled in a model in which epigenetic states are stabilised by a self-perpetuating cycle of interactions between DNA methylation and histone methylation (Figure 1.1).

A link between DNA methylation and chromatin structure was also demonstrated by the identification of the *Arabidopsis* gene *DECREASE IN DNA METHYLATION 1* (*DDM1*) as a member of the yeast ATPase SWI2/SNF2 family of chromatin remodelling machines

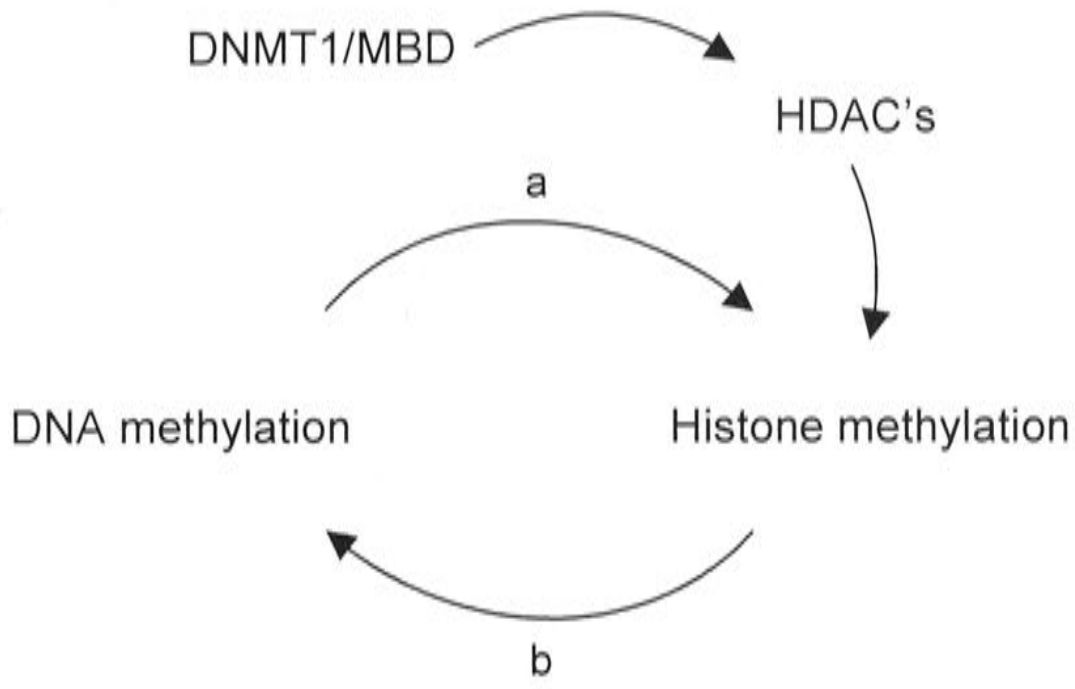


Figure 1.1 DNA methylation and histone methylation may interact in a self-perpetuating cycle to stabilise epigenetic states. HDAC's remove acetyl groups from lysine residues in histones, allowing them to be methylated by histone methyltransferases (Bird, 2001); the murine DNA methyltransferase Dnmt1 can complex with HDAC's (Fuks et al., 2000) and methyl-binding proteins (Tatematsu et al., 2000).

[a] Methylation at CG sequences is proposed to direct methylation of histone H3 in *Arabidopsis* (Soppe et al., 2002).

[b] Replacement of the lysine 9 residue on histone H3 in *Neurospora* (Tamaru and Selker, 2001) or mutation in the histone H3 methyltransferase KYP in *Arabidopsis* causes loss of CNG DNA methylation (Jackson et al., 2002).

(Jeddeloh *et al.*, 1999; Brzeski and Jerzmanowski, 2003). The *ddm1* mutant was isolated on the basis of a reduction in DNA methylation, with the mutant ddm1 protein thought to block access of methyltransferases to heterochromatin (Jeddeloh *et al.*, 1999). DDM1 is also required to maintain methylation of histone H3 (Gendrel *et al.*, 2002). The isolation of the chromodomain-containing *CMT* enzymes (Henikoff and Comai, 1998) suggested that DNA methylation could be targeted to specific regions of chromatin. The realisation that the methyltransferase DNMT1 can complex with a HDAC (Fuks *et al.*, 2000) and can interact with the methyl binding proteins MBD2 and MBD3 (Tatematsu *et al.*, 2000), which also form part of HDAC complexes, further suggested an intimate relationship between DNA methylation and chromatin modification. The association of DNMT1 both with the sliding PCNA factor (Chuang *et al.*, 1997) and with the HDAC/MBD complex suggests that the entire complex could act as a scanning mechanism that searches chromatin for sites to be methylated (Tatematsu *et al.*, 2000).

1.5.2 Methylation and development

The lethal phenotype of *Dnmt1*-deficient mouse embryos (Li *et al.*, 1992) demonstrated unequivocally the importance of DNA methylation in mammalian development, although the actual reason for the lethality still remains to be determined. A wave of demethylation occurs during the blastocyst stage of mammalian embryo development, but the methylation pattern is restored by *de novo* methylation after implantation of the embryo (reviewed in Razin and Cedar, 1993). This global demethylation and remethylation process does not occur during plant embryo development. The rate of remethylation in *ddm1* mutant plants with low methylation levels backcrossed to wild type plants is extremely slow (Kakutani *et al.*, 1999). Similarly, in *Arabidopsis* plants that have low methylation levels due to introduction of an antisense construct against *METI*,

remethylation is slow in subsequent generations of antisense-null segregants (Finnegan *et al.*, 1996). Both DDM1 and METI appear to be required to maintain lysine 9 methylation of histone H3 (Gendrel *et al.*, 2002; Soppe *et al.*, 2002). If histone methylation acts as a signal for DNA methylation (Figure 1.1), the resulting loss of histone methylation in *ddm1* mutants and *METI* antisense plants might prevent remethylation of DNA.

Plants with 10 % of normal CG methylation levels remain viable (Finnegan *et al.*, 1996), in contrast to *Dnmt1*-deficient mice that have 30 % of normal methylation levels (Li *et al.*, 1992). This indicates that DNA methylation in plants could have a different role than in mammals, at least with regards to embryonic development. Nevertheless, the phenotype of plants in which methylation is substantially perturbed has demonstrated that methylation plays important roles in aspects of plant development.

Mutations in the *DDM1* gene cause an immediate loss of methylation in repeated DNA sequences, with a progressive loss of methylation in single copy sequences occurring over several generations of inbreeding (Vongs *et al.*, 1993; Kakutani *et al.*, 1996). The *ddm1* mutant has a reduction in methylation of about 70 % compared to wild type plants. In the first generation of *ddm1* mutant plants, no visible abnormalities were observed (Vongs *et al.*, 1993). However, a progressive loss of methylation in specific genomic regions occurred upon inbreeding these plants (Kakutani *et al.*, 1995, 1996). This was associated with the appearance of a wide spectrum of phenotypic abnormalities, some of which have been attributed to heritable epimutations at several different loci. Defects in the repeatedly selfed *ddm1* mutants included reduced apical dominance, partial sterility, floral abnormalities and delayed flowering (Kakutani *et al.*, 1995, 1996).

In contrast to *ddm1* mutants, plants transformed with a *METI* antisense construct (anti-methyltransferase, AMT), which had up to a 90 % reduction in CG methylation levels, were affected in both single copy and repeated sequences simultaneously (Finnegan *et al.*, 1996; Ronemus *et al.*, 1996). AMT plants showed developmental defects immediately, with the range of abnormalities including reduced apical dominance, decreased height, altered leaf morphology, decreased fertility and altered flowering time. Ectopic expression of the floral homeotic genes *AGAMOUS (AG)* and *APETALA3* (*AP3)* in leaf tissue was also observed (Finnegan *et al.*, 1996). Ectopic expression of the *AG* gene in flowers of some AMT plants resulted in a phenotype resembling that of the *curly leaf (clf)* mutant, in which *AG* and *AP3* are ectopically expressed (Goodrich *et al.*, 1997). *CLF* is homologous to the Polycomb group (PcG) group genes that are involved in the regulation of homeotic gene expression in *Drosophila*. PcG genes establish and maintain the silent state of homeotic genes in groups of cells throughout development, via effects on higher order chromatin structure (reviewed in Pirotta, 1998). PcG proteins are able to methylate histone proteins, thus contributing to the permanently silenced states of homeotic genes (Cao *et al.*, 2002). The identification of *CLF* as a PcG gene, combined with the phenotypic similarities of *clf* mutants to AMT plants, further strengthens not only the link between methylation and chromatin, but also the link between methylation and plant development.

1.5.3 DNA demethylation

The mechanisms controlling the wave of demethylation that precedes *de novo* methylation in the developing mammalian embryo (reviewed in Razin and Cedar, 1993) are yet to be elucidated. Although this process does not occur in plants, methylation levels do change during plant development; for example, seed methylation levels are higher than those of mature tissues or pollen (Messeguer *et al.*, 1991; Palmgren *et al.*, 1991), indicating that

some form of demethylation must occur naturally. One suggested role for demethylation is that of an epigenetic repair system involving targeted demethylation of aberrantly methylated sequences, in order to restore their function (Qu and Ehrlich, 1999).

Methylation could be lost either passively, via lack of maintenance methylation activity during successive cell divisions, or actively, via some direct demethylating activity (reviewed by Wolffe *et al.*, 1999). Three types of demethylating activity have been described. One of these involves the removal of a methylated cytosine by a 5-methylcytosine DNA glycosylase, followed by the replacement of an unmethylated cytosine via an endonuclease activity (Jost *et al.*, 1995). The glycosylase is associated with a RNA moiety that is thought to guide the enzyme to the site that is to be demethylated (Jost *et al.*, 1997). A second report also describes removal of methylated DNA nucleotides, followed by their conversion to RNAse-sensitive forms (Weiss *et al.*, 1996). Demethylase activity of an enzyme identical to the methyl-binding protein MBD2 has been reported (Bhattacharya *et al.*, 1999). The demethylase lacked glycosylase activity, and was instead thought to hydrolyse the 5mC to cytosine and methanol, leaving the DNA strand unperturbed (Ramchandani *et al.*, 1999). However, subsequent reports of the isolation of MBD2 were unable to confirm the claims of demethylase activity (Ng *et al.*, 1999; Wade *et al.*, 1999), and the matter has yet to be resolved.

1.5.4 Methylation and silencing

The silencing of gene expression, first observed in transgenic plants, is now widely accepted as a system that both regulates the expression of endogenous genes and acts as a defence against foreign DNA and viruses. Gene silencing can be broadly separated into two types; silencing dependent on position effects, and silencing dependent on

homology between nucleic acid sequences (homology-dependent gene silencing; Meyer and Saedler, 1996). The latter is traditionally further separated into transcriptional gene silencing, in which the production of RNA transcripts is inhibited, and post-transcriptional gene silencing, which operates at the level of RNA turnover in the cytoplasm. Both TGS and PTGS are associated with *de novo* methylation of homologous sequences, with recent work indicating that the two processes may be intimately linked and not so easily separated (reviewed in Matzke *et al.*, 2001).

1.5.4.1 Position effect silencing

Position effect silencing was first observed in *Drosophila*, where chromosomal rearrangements that placed actively expressed genes next to condensed heterochromatin resulted in their transcriptional inactivation (Reuter and Spierer, 1992). Similar observations were made in transgenic petunia plants (Pröls and Meyer, 1992). In a line in which two transgenes were silenced, the transgenes were found to have integrated into a highly methylated region of DNA, and in two other lines, the different methylation levels of DNA flanking the transgenes in each line impacted on the expression level of the transgenes. This demonstrated that the transcriptional status of the surrounding chromatin region could be imposed upon inserted genes, resulting in silencing if the transgene inserted into a region of heterochromatin (Pröls and Meyer, 1992).

1.5.4.2 Transcriptional gene silencing (TGS)

TGS depends on homology in the promoter region and is correlated with methylation of promoter sequences (Matzke *et al.*, 1989; Meyer *et al.*, 1993), with methylation thought to block transcription via formation of repressive chromatin structures. Methylated transcriptionally silent states are usually meiotically inherited (Meyer *et al.*, 1993; Jones *et al.*, 2001).

In *Neurospora*, TGS can occur via the process of repeat-induced point mutation (RIP) (Selker, 1997; Selker, 1999). In this process, point mutations and DNA methylation occur in duplicated sequences, leading to gene inactivation. Although methylation was found to block elongation of transcripts in *Neurospora* (Rountree and Selker, 1997), RIP is unaffected in the *dim-2* methylation-deficient mutant, bringing into question the role of methylation in this process (Kouzminova and Selker, 2001). A RIP-related process known as methylation induced pre-meiotically (MIP) occurs in *Ascobolus*. MIP is very similar to RIP, but does not involve mutations (Rossignol and Faugeron, 1994). Both RIP and MIP have been proposed to involve DNA-DNA pairing of homologous sequences.

In some cases, endogenous genes silenced by TGS can transmit their silenced state to other alleles. One example is that of paramutation, in which one allele, designated the paramutagenic allele, induces a heritable reduction in expression at a second, paramutable allele. Paramutation has been observed in maize genes involved in anthocyanin biosynthesis pathways, where significant changes in methylation have been correlated with paramutation at the *r1* allele, but not at the *b1* or *pl1* loci. However, it is not known whether methylation plays a direct role in paramutation or is a consequence of it (reviewed in Chandler *et al.*, 2000). The maize *A1* gene caused a paramutation-like phenomenon in transgenic petunia (Meyer *et al.*, 1993) where it was speculated that DNA-DNA pairing was involved.

In plants, methylated, silent loci are thought to be able to induce TGS of other homologous transgenes or endogenous genes via *de novo* methylation of their promoters (Vaucheret, 1993; Park *et al.*, 1996). As for the RIP and MIP processes in fungi, DNA-DNA pairing was originally proposed to account for this *trans*-acting effect (reviewed in Muskens, 2000);

however, little direct evidence of this mechanism exists. Instead, RNA-DNA interactions have been shown to be specific signals for *de novo* methylation (Wassenegger *et al.*, 1994; Pelissier *et al.*, 1999).

Double-stranded RNA (dsRNA) that is produced from inverted repeat transgenes can be cleaved into small RNAs (Hamilton and Baulcombe, 1999). If the dsRNA consists of promoter sequences, the small RNAs can induce promoter methylation and TGS of previously active transgenes or endogenous genes with homologous promoters (Mette *et al.*, 1999, 2000; Sijen *et al.*, 2001; EJ Finnegan and J Dedic, personal communication). This process is thought to involve RNA-directed DNA methylation (RdDM) of complementary DNA sequences (Wassenegger *et al.*, 1994). It is not yet fully understood how RdDM arises, but it is thought that RNA-DNA hybrids may act as templates for methyltransferase enzymes in much the same way that unusual DNA structures are proposed to act (Smith, 1998; Pelissier *et al.*, 1999). A methyltransferase that recognizes RNA-DNA hybrids as a substrate has not yet been unequivocally identified, although CMTs could be potential candidates (Mette *et al.*, 2000; Wassenegger, 2000). It has also been suggested that the small RNAs might guide methyltransferases to homologous DNA sequences, and that RNA helicases may be potential candidates for part of the RdDM machinery (reviewed in Matzke *et al.*, 2001). The chromodomains of histone acetyltransferases, which are part of chromatin-remodelling complexes, have been shown to bind RNA (Akhtar *et al.*, 2000) and small RNAs have recently been implicated in heterochromatinisation of repeat sequences in yeast (Volpe *et al.*, 2002). It is therefore possible that the small RNAs guide chromatin-remodelling machinery to specific chromosomal sites, and that chromatin changes precede RdDM. This is consistent with the observation that the chromodomain-containing methyltransferase CMT3 interacts with

methylated histones via a chromatin binding protein (Jackson *et al.*, 2002), indicating that CMT3 methylates DNA after histone methylation has occurred.

The isolation of mutants with reduced silencing capacity has identified some of the genes required for TGS (reviewed in Mittelsten Scheid and Paszkowski, 2000). The *ddm1* and *hog1* mutants relieve TGS in association with a decrease in methylation (Furner *et al.*, 1998), strengthening the evidence that methylation is necessary for TGS. The methyltransferase METI is essential for the maintenance of TGS. In experiments where *METI* was silenced, TGS of a GFP transgene was reversed, in association with hypomethylation of DNA (Jones *et al.*, 2001). Likewise, in the *met1* mutant, TGS of a GUS transgene was relieved (Morel *et al.*, 2000). In contrast, the *sil* (Furner *et al.*, 1998) and *mom* (Amadeo *et al.*, 2000) mutants relieve silencing without a change in methylation, indicating that more than one pathway to TGS may exist.

1.5.4.3 Post transcriptional gene silencing (PTGS)

PTGS (also called cosuppression) was first observed when endogenous genes were silenced in response to the introduction of homologous transgenes transcribed in the sense direction (Napoli *et al.*, 1990). PTGS, which involves degradation of mRNA in the cytoplasm, is thought to have evolved as a defence against viral invasion and other foreign nucleic acids (Waterhouse *et al.*, 1999, 2001) and has been shown to be equivalent to the RNAi silencing process that occurs in animals (Fire *et al.*, 1998).

Unlike TGS, PTGS requires homology in the transcribed region of genes (Ingelbrecht *et al.*, 1994; reviewed in Depicker and Montagu, 1997; Kooter *et al.*, 1999). However, like TGS, PTGS is associated with dsRNA. DsRNA can be produced from inverted repeat transgenes (e.g. Waterhouse *et al.*, 1998; Mette *et al.*, 2000), by RNA viruses (Ratcliff *et*

al., 1997), or by an RNA-dependent RNA polymerase (RdRP) that uses aberrant single stranded RNA as a template to transcribe complementary RNAs (Wassenegger and Pelissier, 1998). RdRPs have recently been identified in *Neurospora* (Cogoni and Macino, 1999) and *Arabidopsis* (Dalmay *et al.,* 2000; Mourrain *et al.,* 2000). Current models of PTGS (Waterhouse *et al.,* 1998; Wassenegger and Pelissier, 1998; Finnegan *et al.,* 2001; Matzke *et al.,* 2001) hold that the dsRNA molecules are bound and cleaved by a ribonuclease protein complex into small oligomers. The short dsRNAs, while bound to an RNA-induced silencing complex, can then go on to cleave homologous ssRNAs to cause the PTGS phenomenon. The silencing complex, termed RISC in *Drosophila*, comprises an enzyme with nuclease activity that incorporates the dsRNAs as guides to target specific messages (Hammond *et al.,* 2000). In plants, the same short dsRNA/protein complex is also capable of inducing RdDM, resulting in dense methylation at both symmetrical and non-symmetrical sites in the transcribed regions that are homologous between the RNA and DNA, just as occurs in promoter regions in TGS (Jones *et al.,* 1999; Pelissier *et al.,* 1999; Sijen *et al.,* 2001). The PTGS signal can spread to other cells, as demonstrated by grafting experiments (Palauqui *et al.,* 1997) and the signal is amplified as it moves through the plant (Voinnet *et al.,* 1998). The signal is yet to be identified but the short dsRNA/protein complex described above is one candidate, as it might be able to travel to other cells, where it could propagate RNA degradation as well as direct sequence-specific RdDM (Wang *et al.,* 2001).

A reduction in DNA methylation in several *Arabidopsis* RdRP mutants suggested that establishment or maintenance of PTGS might require methylation (reviewed in Matzke *et al.,* 2001). A role for methylation and chromatin modification in both the establishment and maintenance of PTGS was suggested by the reactivation of a post-transcriptionally silenced GUS transgene in the presence of *ddm1* and *met1* mutations (Morel *et al.,* 2000).

However, virus-induced gene silencing experiments showed that METI is not required for either maintenance of PTGS or initiation of RdDM (Jones *et al.*, 2001), indicating that different methyltransferase/s, possibly RNA-directed methyltransferases, may be involved in these functions. Jones et al. (2001) suggest that METI-dependent and METI-independent systems of PTGS maintenance might exist, to account for the variation in correlations between PTGS and methylation.

1.5.5 Regulation of endogenous gene expression by methylation

The expression of several endogenous *Arabidopsis* genes appears to be regulated by DNA methylation. Analysis of the *PAI* (phosphoribosylanthranilate isomerase) genes in the WS ecotype revealed four highly similar *PAI* genes at three loci; *PAI1-PAI4*, which is an inverted repeat, and *PAI2* and *PAI3,* found at unlinked loci. All four genes are heavily methylated (Bender and Fink, 1995). *PAI1* is active, thought to be due to transcription from a promoter in a upstream unmethylated region (Melquist *et al.,* 1999), but *PAI2* and *PAI3* are silenced. Deletion of the inverted repeat *PAI* genes resulted in a loss of methylation and expression of the other two genes, suggesting that the inverted repeat genes promoted methylation of the others. This observation was supported by the fact that the *PAI* genes are unmethylated in the Columbia (Col) ecotype, which does not have the inverted repeat genes. In crosses between WS and Col, the inverted repeat genes of WS triggered *de novo* methylation of the Col genes (Luff *et al.,* 1999), and in a screen of 39 other ecotypes, the presence of the inverted repeat *PAI* genes correlated with methylation of all the *PAI* genes (Melquist *et al.,* 1999). Crossing the *PAI* lines to *ddm1* and antisense *METI* lines led to a reduction in methylation and reactivation of *PAI2* (Jeddeloh *et al.,* 1998; Bender, cited in Jacobsen, 1999) and a genetic screen for reduced *PAI* methylation

resulted in isolation of a *CMT3* mutant (Bartee *et al.*, 2001), further strengthening the link between methylation and silencing of the *PAI* genes.

Other endogenous genes whose expression is regulated by methylation include *SUPERMAN (SUP), AGAMOUS (AG)* and *FWA*. In contrast to the *PAI* genes, the *SUP* gene is unmethylated in wild-type plants, but epialleles of *SUP* that arose in mutagenised plants were densely methylated and transcriptionally silenced (Jacobsen and Meyerowitz, 1997). The *SUP* gene becomes hypermethylated and silenced in the low methylation background of AMT plants (Jacobsen and Meyerowitz, 1997). Neither DDM1 or METI are required for establishment or maintenance of *SUP* hypermethylation (Jacobsen *et al.*, 2000), which is concentrated within CNG sites, suggesting the involvement of CMT enzymes (Jacobsen and Meyerowitz, 1997). AMT plants also show a phenotype resembling the floral homeotic mutant *ag* (Finnegan *et al.*, 1996), due to methylation and silencing of the *AG* gene. Most of the methylation in the *AG* sequence is asymmetric, suggesting a methyltransferase other than *METI* was responsible (Jacobsen *et al.*, 2000). Both the *SUP* and *AG* genes contain pyrimidine-rich sequences that are predicted to form secondary hairpin structures which can be preferentially methylated, acting as a potential signal for their methylation and silencing (Jacobsen *et al.*, 2000). *FWA* is normally silenced in wild type plants, correlating with the methylation of two direct repeat sequences in its 5' region; expression of *FWA* is associated with hypomethylation of about 5 Mb around the *FWA* locus (Soppe *et al.*, 2000). As methylation within *FWA* is not confined to CG sites but also occurs at asymmetric sites, it has been proposed that the silencing of *FWA* could be a result of RdDM, with the 5' repeated sequences potentially producing dsRNA (Soppe *et al.*, 2000). This indicates that similar processes could regulate the expression of both transgenes and endogenous genes.

Endogenous TGS has also been observed in the naturally occurring *Lcyc* mutant, identified in natural populations of *Linaria vulgaris* (Cubas *et al.*, 1999). Identification of this mutant, in which the *Lcyc* gene is methylated and silenced, has suggested a role for the epigenetic regulation of genes in evolution (reviewed in Finnegan, 2002).

1.5.6 Methylation and genome defence

One of the major roles posited for methylation is as the basis of a nuclear defence system that protects the genome against potential deleterious effects of parasitic sequence elements such as transposable elements (TE's), viral and viroid DNA (Yoder *et al.*, 1997). Both plant and animal genomes contain a large number of TE's and proviral DNA sequences, which are normally methylated and silent (Bestor, 1990; Bestor and Coxon, 1993; Bestor and Tycko, 1996). The activation of parasitic sequence elements in response to demethylation can wreak havoc, for instance, causing ectopic expression of adjacent genes (reviewed in Yoder and Bestor, 1996). In mice, demethylation of an intracisternal A retrovirus leads to overexpression of the adjacent *agouti* gene, causing gross growth abnormalities (Michaud *et al.*, 1994). In a mammalian interspecific hybrid, activation of mobile elements by demethylation leads to rapid genome alterations and even changes in chromatin structure (Waugh O'Neill *et al.*, 1998), indicating that methylation has an important role in suppressing these elements.

In plants, transposon and retrotransposon activity also appears to be regulated by methylation and chromatin structure (e.g. Schwartz and Dennis, 1986). The methyltransferase CMT3 is required to maintain gene silencing of retrotransposon sequences (Lindroth *et al.*, 2001), and some of the epimutations seen in *ddm1* mutant plants are due to demethylation and activation of transposons. For instance, in the

absence of *DDM1* function, the *CAC1* transposon becomes demethylated, transposes and increases in copy number. Insertion of *CAC1* into *DWF4*, a brassinosteroid biosynthesis gene, results in a lack of shoot and petiole elongation, causing the *clam* abnormality (Miura *et al.*, 2001). Robertson's *Mutator*-like elements (Singer *et al.*, 2001) and *Athila4* retroelements (Wright and Voytas, 2001) also become demethylated and activated in *ddm1* plants. Similarly, the tobacco *Tto1* retrotransposon becomes methylated and silenced when inserted into *Arabidopsis* and then is demethylated and activated in the *ddm1* background (Hirochika *et al.*, 2000). These observations indicate that methylation and chromatin remodelling are required for transcriptional repression of many transposons. DsRNA produced from repetitive transposon sequences could act as a signal for RdDM, leading to silencing and inactivation of the transposons (reviewed in Muskens et al, 2000), thus linking genome defence and gene silencing mechanisms. It has been suggested that many epigenetic phenomena could be a consequence of the endogenous genome defence system (Henikoff and Matzke, 1997).

1.5.7 Methylation and genomic imprinting

The expression status of some alleles is dependent on the parent from which they were inherited. This phenomenon, known as genomic imprinting, involves one of the two alleles of particular genes being suppressed throughout development. Imprinting has often been correlated with methylation and/or chromatin-mediated silencing (reviewed in Alleman and Doctor, 2000). The concept of genomic imprinting is not solely restricted to the definition of parental origin; the term can also cover heritable changes brought about by interactions between alleles such as paramutation (reviewed in Matzke and Matzke, 1993).

In mice, three imprinted genes are differentially methylated, depending on their parental origin. The proper differential expression of these three alleles is compromised in Dnmt1-deficient mice (Li *et al.,* 1993). Transformation of mutant *dnmt1* embryonic stem cells with a wild type *Dnmt1* construct re-established normal methylation levels in all non-imprinted genes, but failed to restore normal methylation in imprinted genes, indicating that a different methyltransferase may be required to methylate imprinted genes (Tucker *et al.,* 1996). The identification of alternatively spliced forms of Dnmt1 that are active at different stages of development and in different places led to the suggestion that one form of Dnmt1 was responsible for *de novo* methylation in eggs (Mertineit *et al.,* 1998), but more recent results indicate that it is needed for maintenance methylation only in the embryo (Howell *et al.,* 2001). An protein known as Dnmt3L, which is homologous to the *de novo* Dnmt3 enzymes, is required specifically for the establishment of genomic imprinting in female gametes. The mechanism of how this occurs is unclear, as Dnmt3L lacks the characteristic catalytic motifs of methyltransferases (Bour'chis *et al.,* 2001).

In plants, parental imprinting has been observed in the seed endosperm, which in angiosperms, contains one paternal and two maternal genomes (reviewed in Matzke and Matzke, 1993). The maize *R* gene, which encodes a transcription factor involved in anthocyanin production, is imprinted in the endosperm. The maternally inherited allele is undermethylated compared to the paternal allele, with inheritance from the maternal or paternal side resulting in different pigmentation patterns (Kermicle and Alleman, 1990). Endosperm development and the control of seed size in *Arabidopsis* also appears to be regulated by genomic imprinting, as different ratios of maternal to paternal genomes cause either inhibition or promotion of seed size (Scott *et al.,* 1998). Reciprocal crosses between AMT plants with low methylation levels and wild type plants phenocopies this effect, with AMT pollen giving the effect of having an extra maternal genome, and wild type pollen

creating an extra paternal genome effect (Adams *et al.*, 2000). These observations suggest a general role for methylation in imprinting mechanisms in plants.

The maternally derived allele of the *Arabidopsis MEA* (*FIS1*) gene, which is homologous to the PcG genes of *Drosophila*, is expressed in the endosperm after fertilization, but the paternally derived allele is silenced in sperm cells. Seeds of the *mea* mutant abort, but when *mea* is crossed to *ddm1*, the lethal phenotype is rescued, implying that the paternally-inherited *MEA* allele is reactivated (Vielle-Calzada *et al.*, 1999). This was interpreted to mean that during seed development, the maintenance of the genomic imprint on the paternal *MEA* allele requires the activity of *DDM1,* and hence that changes in chromatin structure (and perhaps methylation) have an effect on genomic imprinting. Mutants of the maternally expressed PcG genes *FIS2* (Luo *et al.*, 2000) and *FIE* (Vinkenoog *et al.*, 2000) were also rescued by a reduction in methylation levels after pollination with AMT plants. However, this is not due to reactivation of the paternal alleles, but probably due to activation of downstream genes, calling into question the role of methylation in maintaining the genomic imprint of *FIS2* and *FIE (*Luo *et al.*, 2000; Vinkenoog *et al.*, 2000), as well as *MEA* (Grossniklaus *et al.*, 2001).

1.5.8 Methylation and large-scale silencing

DNA methylation has been implicated in many other aspects of gene regulation in addition to those already described. One of these is the process of X-chromosome inactivation (XCI), a method of dosage compensation in mammals in which one of the two X-chromosomes is silenced. XCI involves the production of a non-coding RNA called *Xist*, which coats the inactive chromosome and is required for the initiation of the inactivation process (reviewed in Park and Kuroda, 2001). *Xist* is expressed from the inactive X

chromosome, and the silent *Xist* allele on the corresponding active X chromosome is methylated (reviewed in Pfeifer and Tilghman, 1994). Demethylation of the *Xist* locus in *Dnmt1*-deficient mouse embryos induces its expression (Beard *et al.*, 1995) and results in silencing of both X chromosomes (Panning and Jaenisch, 1996), demonstrating the importance of methylation in the maintenance of *Xist* expression. It has been suggested that the DNMT3a and/or 3b enzymes could be responsible for *de novo* methylation of *Xist* during normal development (Okano *et al.*, 1999).

DNA methylation is also associated with the process of nucleolar dominance (reviewed in Pikaard, 2000). Nucleolar dominance involves the silencing of one parental set of rRNA genes in hybrids. As there are thousands of copies of rRNA genes, this silencing phenomenon occurs on an enormous scale. Although the initiating mechanisms of nucleolar dominance are unclear, the silenced state is thought to be maintained by DNA methylation and repressive chromatin structures, as treatment with inhibitors of DNA methylation and histone deacetylases de-represses silent rRNA genes (Chen and Pikaard, 1997).

1.6 The floral transition

To initiate flowering, plants must perceive and respond to many different signals that are received both from the external environment and from within the plant. Important environmental signals influencing flowering include light and temperature, and endogenous signals include hormones such as gibberellic acid (GA). The time to flowering of the crucifer *Arabidopsis thaliana* is significantly reduced by both long day light periods (Napp-Zinn, 1985) and GA (Langridge, 1957). A prolonged exposure to cold temperatures also induces flowering in many annual species, a response termed

vernalisation (Napp-Zinn, 1985). This response confers a reproductive advantage on plants, allowing them to flower as soon as possible after winter.

The process of floral initiation is facilitated by an intricate network of flowering time genes that function in a variety of pathways, each regulated by a different combination of signals. The flowering pathways converge to upregulate floral meristem identity (FMI) genes, followed by genes that specify the development of the floral organs. Two important FMI genes that lie downstream of the flowering time genes are *APETALA1* (*AP1*), a MADS-box transcription factor (Irish and Sussex, 1990) and *LEAFY* (*LFY*), a novel transcription factor (Mandel *et al.*, 1992). Together, *LFY* and *AP1* promote the transition from inflorescence meristem to floral meristem on the flanks of the shoot meristem (Irish and Sussex, 1990; Weigel *et al.*, 1992; Bowman *et al.*, 1993; Mandel and Yanofsky, 1995). To retain inflorescence identity at the centre of the growing tip, their expression is excluded from the centre of the inflorescence meristem by the floral repressor *TERMINAL FLOWER 1 (TFL1)* (Bradley *et al.*, 1997; Ruiz-Garcia *et al.*, 1997). In a reciprocal negative relationship, *LFY* and *AP1* exclude the expression of *TFL1* in the flanks of the shoot meristem (Shannon and Meeks-Wagner, 1993; Ratcliffe *et al.*, 1999).

The identity of the different organs that are generated from floral meristem tissue is specified by the expression of floral organ identity genes. These include *AP1*, *APETALA3 (AP3)*, *PISTILLATA (PI)* and *AGAMOUS (AG)*, which are MADS-box transcription factors (Weigel and Meyerowitz, 1994), as well as *APETALA2 (AP2)*, which belongs to a different class of transcription factor (Jofuku *et al.*, 1994). Differential region-specific expression of these genes in the four whorls of the flower results in the production of sepals, petals, stamens and carpels. Some floral organ identity genes are repressed by plant homologues of the *Drosophila* PcG genes that stably maintain the repression of homeotic genes

throughout development (Simon, 1995). For example, the PcG gene *CLF* represses *AG* throughout the plant except within the inner two whorls of the flower, where *AG* is required for specification of stamens and carpels (Goodrich *et al.*, 1997).

1.7 Natural variation in flowering time

At least 80 genes are thought to be involved in regulating the initial transition from vegetative to reproductive growth (Levy and Dean, 1998). Whilst many of these loci have been identified by mutant analysis, studies of natural variation in flowering time among different ecotypes of *Arabidopsis* have also led to the identification of some important flowering time genes, including *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*). Dominant alleles of *FRI* and *FLC* cause late flowering which is reversible by vernalisation. *FRI* was identified as a single dominant gene causing differences in flowering time between the late ecotype San-feliu 2 (Sf-2) and the early ecotypes Landsberg *erecta* (Ler) and Columbia (Col) (Lee *et al.*, 1993). The failure of the Sf-2 allele to cause late flowering in Ler led to the identification of *FLC*, and suggested that the lateness caused by *FRI* depended on a dominant allele at the *FLC* locus (Koornneef *et al.*, 1994; Lee *et al.*, 1994b; Clarke and Dean, 1994). Although *FLC* and *FRI* are clearly important flowering genes, the identification of many other naturally occurring loci that affect flowering, in crosses between different ecotypes, has demonstrated that differences in flowering behaviour in different genetic backgrounds can not usually be attributed to a single gene (Alonso-Blanco *et al.*, 1998).

1.8 Flowering mutants

Mutation-based studies on flowering, beyond the initial identification of the *ld, gi and co* mutants (Redei, 1962) were greatly extended by the work of Koornneef *et al.* (1991), who identified 11 different flowering loci. By 1998 studies of these and other mutants, along with naturally occurring loci (Koornneef *et al.* 1991, 1998b; Lee *et al.,* 1993; Martinez-Zapater *et al.,* 1994) resulted in the identification of many more flowering loci. Together, these studies led to a general model defining flowering as a default developmental state (Koornneef *et al.,* 1998a; Simpson *et al.,* 1999; Reeves and Coupland, 2000), although it has also been suggested that flowering might instead be actively switched on by genes such as *LFY* (Blazquez and Weigel, 2000).

The default model was proposed because although mutants without any evidence of floral structures had never been identified, the *embryonic flower (emf)* mutant develops directly into the early reproductive stage immediately after germination (Sung *et al.,* 1992). The default flowering state was therefore suggested to be suppressed by floral repressors such as *EMF1*, with early and late flowering mutants representing genes that either repress or promote the default flowering state in wild type plants (Koornneef *et al.,* 1998a). These mutants, along with the naturally occurring loci, act within four generally accepted floral promotion pathways; the endogenously regulated autonomous and GA-mediated pathways, and the environmentally regulated photoperiod and vernalisation pathways (Figure 1.2). Although each pathway can independently result in flowering, many overlaps and interactions also exist between them (see section 1.13).

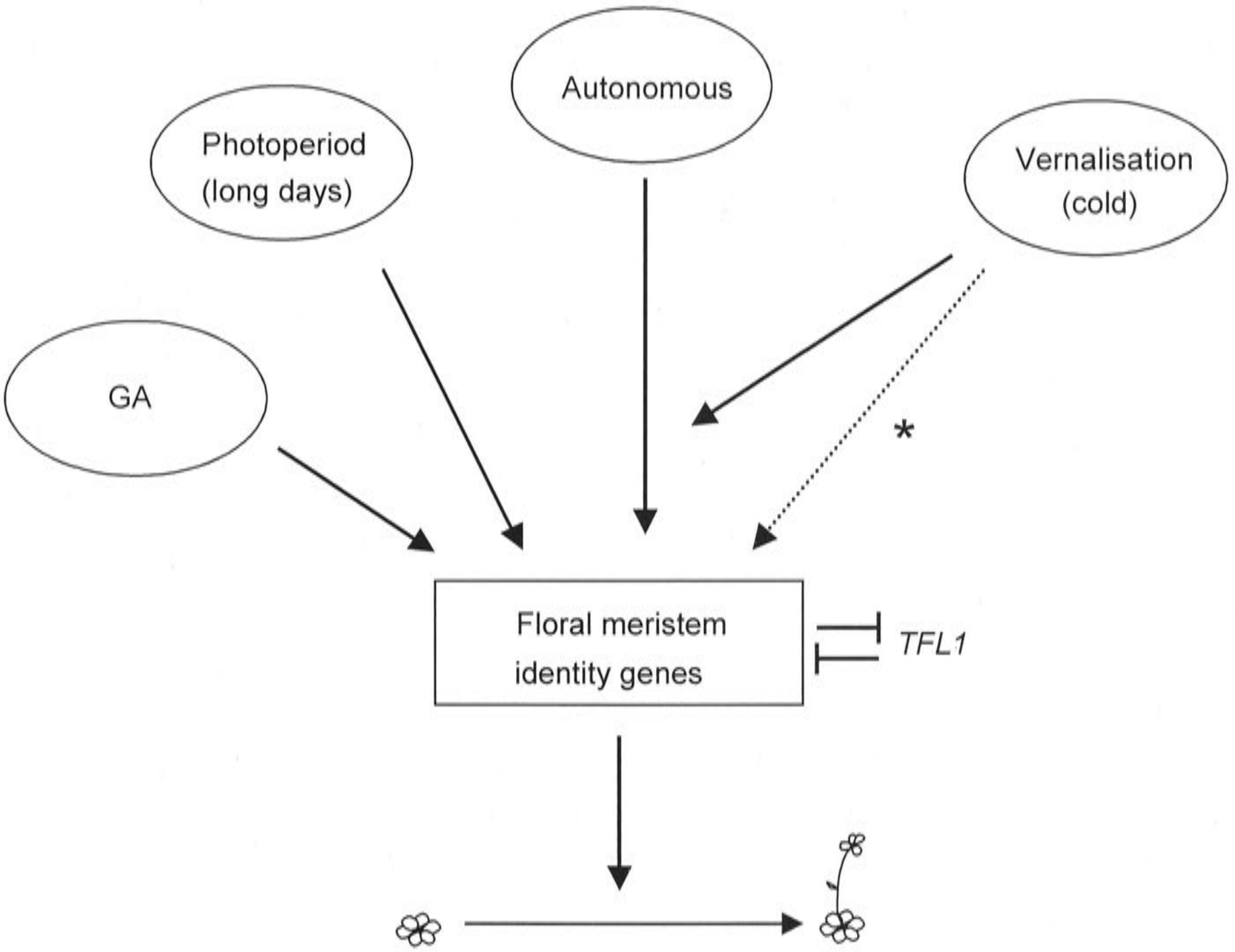


Figure 1.2. Floral promotion pathways in *Arabidopsis thaliana*.

* *FLC*-independent pathway to flowering (Michaels and Amasino, 2001; Reeves and Coupland, 2001)

1.9 Photoperiod promotion pathway

Mutants in the photoperiod promotion pathway lose their sensitivity to daylength, flowering at the same time as wild type plants in short days (SD), but later than wild type in long days (LD) conditions. They also have a reduced response to vernalisation relative to wild type plants. Late flowering mutants placed in this pathway include *co, gi, fd, fha, fe, ft, fwa* and *soc1* (Koornneef *et al.,* 1998a; Kardailsky *et al.,* 1999; Kobayashi *et al.,* 1999; Onouchi *et al.,* 2000; Samach *et al.,* 2000).

The *CONSTANS (CO)* and *GIGANTEA (GI)* genes lie downstream of a light sensing mechanism, which consists of light-sensitive photoreceptors and circadian clock components (Koornneef and Peeters, 1997). *GI* encodes a putative membrane-localised protein (Fowler *et al.,* 1999; Park *et al.,* 1999), thought to form part of a feedback loop that controls light signalling to the circadian clock upstream of *CO* (Igeno, Robson and Coupland, cited in Fowler *et al.,* 1999; Park *et al.,* 1999). *CO* shows resemblance to zinc-finger transcription factors (Putterill *et al.,* 1995), with the zinc finger domain being homologous to the "B-box" protein-protein interaction domains of animal transcription factors (Robson *et al.,* 2001). CO promotes flowering in a dosage-dependent manner (Putterill *et al.,* 1995) and its overexpression results in photoperiod-independent early flowering (Simon *et al.,* 1996). However, CO also has antagonistic effects on flowering, as it leads to activation of both the floral repressor *TFL1* and the floral promoter *LFY* (Simon *et al.,* 1996; Bradley *et al.,* 1997).

Although the four mutants *ft, fwa, fe* and *fd* have the general characteristics of photoperiod pathway mutants (Koornneef *et al.,* 1991), they are generally placed as a separate subgroup. Unlike most other flowering genes, genes of this subgroup do not upregulate

expression of the FMI gene *LFY* (Nilsson *et al.,* 1998), but instead control the competence of the meristem to respond to *LFY* expression (Ruiz-Garcia *et al.,* 1997). *FT* is in the same gene family as *TFL1*, both genes sharing similarities with membrane-associated proteins involved in signal transduction (Kardailsky *et al.,* 1999; Kobayashi *et al.,* 1999). *FWA* encodes a homeodomain-containing transcription factor (Soppe *et al.,* 2000). CO promotes flowering via *FT* and *FWA* (Onouchi *et al.,* 2000), with *FT* and a putative transcription factor, *SUPPRESSOR OF OVEREXPRESSION OF CO 1 (SOC1)*, being direct early target genes of CO (Onouchi *et al.,* 2000; Samach *et al.,* 2000). *FT* is also thought to be upregulated by a *CO*-independent pathway, because although its expression is delayed in the *co* mutant, it does eventually occur (Kardailsky *et al.,* 1999; Kobayashi *et al.,* 1999).

1.10 Autonomous promotion pathway

Mutants in the autonomous pathway, which include the classical late flowering mutants *fpa, fve, fld, ld, fy* and *fca,* are late flowering in LD and SD, and are responsive to vernalisation (Koornneef *et al.,* 1991, 1998b). Within this class of mutants, *fca* and *fy* form one subgroup, and *fve* and *fpa* form a second (Koornneef *et al.,* 1998; Rouse *et al.,* 2002). The autonomous pathway genes promote flowering independently of environmental signals, but possibly respond to endogenous signals, such as signals from an internal developmental clock (Simpson *et al.,* 1999).

Of the autonomous pathway genes, *FPA, FCA, LD* and *FY* have been cloned. *FPA* and *FCA* contain RNA-binding motifs, indicating possible involvement in post-transcriptional events (Macknight *et al.,* 1997; Schomburg *et al.,* 2001). Overexpressing *FPA* causes early flowering in SD, thought to be due to either negative regulation of floral repressors, or

interaction with photoperiod pathway genes (Schomburg *et al.*, 2001). *FCA* overexpression also causes early flowering, probably via upregulation of *LFY* (Page *et al.*, 1999). In addition to its two RNA binding domains, FCA contains a protein interaction domain, further suggesting that it acts at a post-transcriptional level (Macknight *et al.*, 1997). Consistent with this, the C-terminal region of FCA interacts with a AtSWI3B, a component of a chromatin remodelling complex (Sarnowski *et al.*, 2002). *LD* has a glutamine-rich domain often found in transcription factors (Lee *et al.*, 1994a) and shows similarities to homeodomain DNA-binding proteins of yeast (Aukerman and Amasino, 1996). The *LD* gene product is targeted to the nucleus (Aukerman *et al.*, 1999).

FLC, a MADS-box transcription factor, mediates the action of all the autonomous pathway genes (Michaels and Amasino, 1999a; Sheldon *et al.*, 1999). *FLC* was originally placed in the autonomous pathway because crossing the dominant Columbia *FLC* allele to autonomous pathway mutants such as *fca* and *fpa* caused them to flower even later (Sanda and Amasino, 1996). *FLC* is upregulated by *FRI*, which is not related to any known protein (Johansen *et al.*, 2000). Although *FRI* was originally placed in the autonomous pathway due to similarities in its flowering phenotype with the other autonomous mutants (Martinez-Zapater *et al.*, 1994), it acts independently of the other autonomous genes.

FLC represses flowering in a dosage-dependent manner (Sheldon *et al.*, 1999). It is expressed at a low level in early flowering ecotypes such as Ler and Col that have loss-of-function *FRI* alleles (Johansen *et al.*, 2000). Conversely, *FLC* is highly expressed in late flowering ecotypes with a dominant *FRI* allele (Michaels and Amasino, 1999a; Sheldon *et al.*, 1999); in the absence of *FLC*, such ecotypes lose their late flowering phenotype (Michaels & Amasino 2001). Consistent with the placement of *FLC* within the autonomous

promotion pathway, *FLC* expression is higher in the autonomous mutants *fca, fve, fpa* and *ld* than in the wild type parent (Michaels and Amasino, 1999a; Sheldon *et al.*, 1999), whereas *FLC* is not upregulated in photoperiod pathway mutants (Sheldon *et al.*, 1999). The late flowering phenotype of most of the autonomous pathway mutants appears to be entirely *FLC* dependent, since a null *FLC* allele abolishes the late flowering of all but one of them (Michaels and Amasino, 2001). *FPA* is the exception, retaining some late flowering effect, in keeping with its placement in between the photoperiod and autonomous pathways (Koornneef *et al.*, 1998b).

1.11 Vernalisation promotion pathway

The ability to respond to vernalisation provides a reproductive advantage for annual plants such as *Arabidopsis* by allowing flowering to occur in spring weather, thus providing ample time to set seed before the onset of the following winter. The capacity of different ecotypes of *Arabidopsis* to respond to vernalisation is largely determined by the presence of dominant alleles of *FRI* and *FLC* (Napp-Zinn, 1985; Lee *et al.*, 1993, 1994b; Koornneef *et al.*, 1994).

The basis of the molecular mechanism of the early flowering response to vernalisation is the down-regulation of *FLC* expression (Sheldon *et al.*, 1999). The level of *FLC* expression is high in the late-flowering vernalisation-responsive mutants *fca, fve, fpa* and *ld* and in late flowering ecotypes such as Pitztal; conversely, vernalisation non-responsive ecotypes and mutants have low levels of *FLC* expression (Michaels and Amasino, 1999a; Sheldon *et al.*, 1999). The quantitative nature of the vernalisation response described some years ago (Martinez-Zapater and Somerville, 1990) can be explained by the extent of decrease in *FLC* expression levels, which is proportional to the length of the vernalisation period

(Michaels and Amasino, 1999a; Sheldon *et al.*, 1999). Plants that overexpress *FLC* require longer vernalisation periods than normal to flower early, further demonstrating the importance of *FLC* as a key regulator of the vernalisation response (Sheldon *et al.*, 1999). However, an *FLC*-independent vernalisation pathway has also been proposed to exist, because after a 70 day vernalisation treatment, null *flc* plants flower with half the number of leaves of unvernalised plants (Michaels and Amasino, 2001).

The recently identified *VIP4 (VERNALISATION INDEPENDENCE 4)* gene is a positive regulator of *FLC* expression. In the unvernalised *vip4* mutant, *FLC* expression is decreased, causing early flowering (Zhang and van Nocker, 2002). However, the expression of *VIP4* is not affected by vernalisation, demonstrating that *FLC* suppression does not result from *VIP4* down-regulation and suggesting that *VIP4* is not sufficient to activate *FLC* in vernalised plants (Zhang and van Nocker, 2002).

Mutants which have a reduced response to vernalisation have provided further clues to the vernalisation mechanism. The *vrn1* and *vrn2* mutants were isolated in the late flowering vernalisation-responsive autonomous mutant *fca* (Chandler *et al.*, 1996). *VRN1* encodes a nuclear-localised DNA-binding protein (Levy *et al.*, 2002) and *VRN2* encodes a nuclear-localised zinc finger protein that is homologous to the *Drosophila* Polycomb Group (PcG) protein Suppressor of Zeste 12 (Su(Z)12) (Gendall *et al.*, 2001). Both *VRN1* and *VRN2* are required for the post-vernalisation stable repression of *FLC* throughout development, but not for its initial decrease (Gendall *et al.*, 2001; Levy *et al.*, 2002). Overexpression of *VRN1* causes early flowering, not by decreasing *FLC* expression but by up-regulating *SOC1* and *FT* expression (Levy *et al.*, 2002), indicating that *VRN1* has multiple roles in promoting flowering.

A gene which responds positively to vernalisation has also been identified. *EARLI1* encodes a small hydrophobic proline-rich protein (Wilkosz and Schlappi, 2000). The expression of the *EARLI1* gene is inversely correlated to that of *FLC*, but it is not known whether *FLC* represses *EARLI1*. The expression of *EARLI1* increases both after a vernalisation treatment and in early flowering ecotypes, but it has yet to be determined whether this gene is necessary for vernalisation (Wilkosz and Schlappi, 2000).

1.12 GA promotion pathway

The plant hormone gibberellic acid (GA) promotes flowering in many species, even in non-inductive conditions (Zeevart, 1983), and has long been known to be important in the promotion of flowering in *Arabidopsis* (Langridge, 1957). The GA promotive pathway is usually placed as a pathway parallel to the other three flowering pathways. The application of exogenous GA decreases the flowering time of all known late flowering mutants (Chandler and Dean, 1994), and combining GA mutants with photoperiod mutants (Putterill *et al.*, 1995) or autonomous mutants (Chandler and Dean, cited in Simpson *et al.*, 1999) yields phenotypes consistent with GA acting in a separate promotive pathway. There are two general types of GA mutants with altered flowering phenotypes; those with mutations in GA biosynthetic genes, and those with mutations in the GA signal transduction pathway downstream of the biosynthesis pathway. Repeated applications of exogenous GA restores normal growth of GA biosynthesis mutants (Koornneef and van der Veen, 1980), but not of GA signal transduction mutants (reviewed in Ross *et al.*, 1997).

The GA biosynthesis mutant *ga1-3* has a deletion in the kaurene synthase gene *GA1* (Koornneef *et al.*, 1983), and as a result, synthesises very little *ent*-kaurene (Sun and Kamiya, 1994), which is an early intermediate in the GA biosynthesis pathway. The *ga1-3*

mutant is extremely dwarfed, flowers very late in LD or under constant light, but does not flower at all in SD (Wilson *et al.,* 1992), demonstrating that GA is absolutely required for flowering in Arabidopsis under SD conditions.

GA signal transduction mutants fall into one of two classes. Mutant of the GA non-responsive dwarf class germinate poorly, have dark green compact leaves and flower significantly later than wild type plants. Such mutants include *gai* (***GA**-**i**nsensitive*; Koornneef *et al.,* 1985) and *shi* (***sh**ort **i**nternodes*; Fridborg *et al.,* 1999). The *gai* mutant has a gain-of-function mutation in a repressor of the GA signal transduction pathway; the mutant gai repressor is insensitive to GA (Peng *et al.,* 1997). The *shi* mutant phenotype results from overexpression of S*HI,* a gene with similarities to zinc-finger transcription factors. Like GAI, SHI is also a negative regulator of GA signal transduction (Fridborg *et al.,* 1999). In contrast to the GA-insensitive mutants, the class of so-called GA "overexpressors" resemble wild type plants treated with excessive GA. These mutants are pale green, have elongated stems and long petioles, and flower earlier than wild type plants. Mutants include *rga* (***R**epressor of the **ga**1-3* mutant; Silverstone *et al.,* 1997) and *spy* (*SPINDLY*; Jacobsen and Olsewski, 1993). The wild type *RGA, RGA* homologues and *SPY* gene products are negative regulators of the GA biosynthesis pathway (Jacobsen and Olsewski, 1993; Jacobsen *et al.,* 1996; Silverstone *et al.,* 1997; Sun, 2000), with their main role being to delay the juvenile-to-adult transition (Dill and Sun, 2001). *RGA* has a high degree of homology to *GAI* (Silverstone *et al.,* 1998), and *SPY* is thought to play a role in the post-translational activation of both *RGA* and *GAI* (Thornton *et al.,* 1999).

The net result of flux through the GA biosynthesis and signal transduction pathway is upregulation of the FMI gene *LFY* (Blazquez *et al.,* 1998). The late flowering phenotype of the *ga1-3* mutant is due to a lack of *LFY* promoter induction (Blazquez *et al.,* 1998), and a

LFY promoter sequence with similarities to *myb* transcription factor binding sites is proposed to act as a GA response element (Blazquez and Weigel, 2000). Another gene which upregulates *LFY* via the GA-mediated pathway to flowering is *FPF1* (*FLOWERING PROMOTING FACTOR 1;* Kania *et al.,* 1997). Overexpression of *FPF1* causes upregulation of *LFY* and early flowering. *FPF1* expression is proposed to enhance the responsiveness of the apical meristem to GA (Kania *et al.,* 1997; Melzer *et al.,* 1999), making the meristem more competent to respond to *LFY* (Melzer *et al.,* 1999).

1.13 Integration of the floral promotion pathways

The more information that is gathered about the four flowering pathways, the more it seems likely that they should be seen not as discrete entities but instead as interacting sections of a complex network. These interactions become apparent when functions of one pathway are revealed only in the presence of a defect in another pathway, or in conditions that affect the operation of another pathway. For example, under SD conditions, when the photoperiod pathway is compromised, the autonomous, GA and vernalisation pathways become relatively more important. This capacity to respond in different ways to different signals gives plants the flexibility to adapt to a wide range of environmental conditions.

Many interactions between the four floral promotion pathways have been identified by the construction of double mutant plants. For instance, the photoperiod mutants *ft, fe* and *fha,* which have wild type levels of *FLC* expression, exhibit an increase in *FLC* levels when combined with the autonomous pathway mutants *fca* and *fpa*, showing that these wild type photoperiod genes can downregulate *FLC* in the absence of functioning autonomous pathway genes (Rouse *et al.,* 2002). Photoperiod mutants also interact with GA signal

transduction mutants, with both *co* and *gi* enhancing the late flowering phenotype of *GAI* mutants (Simon and Coupland, 1996). GI interacts at the protein level with SPY, indicating further crosstalk between the GA and photoperiod pathways (N.E. Olszewski, cited in Meier *et al.*, 2001).

GA accelerates flowering of autonomous pathway mutants such as *fca* (Bagnall, 1992). The autonomous mutant *fpa*, previously proposed to link the autonomous and photoperiod pathways (Koornneef *et al.*, 1998b), was also identified in a screen for GA response mutants (Meier *et al.*, 2001). In the presence of a null *FLC* allele, *fpa* displays a phenotype reminiscent of GA-deficiency (Michaels and Amasino, 2001), indicating further interactions between the GA and autonomous pathways. Interactions have also been proposed to occur between the GA and vernalisation pathways, one example being that of an increase in the metabolism of a GA precursor, kaurenoic acid, in shoot tips of vernalised plants (Hazebroek and Metzger, 1990).

The complicated network of flowering pathways ultimately converges at several points to trigger the initiation of floral structures via activation of *AP1* and floral organ genes (Figure 1.3). The most important points of integration identified to date are at the floral promoters *LFY*, *FT* and *SOC1*. *LFY* expression is upregulated by the photoperiod, autonomous and GA pathways (Simon *et al.*, 1996; Blazquez *et al.*, 1998, Nilsson *et al.*, 1998) and overexpression of *LFY* overcomes the late flowering of the *co, fca, fve* and *gai* mutants (Nilsson *et al.*, 1998). Discrete elements in the *LFY* promoter have been identified as targets of GA, and indirectly, via *SOC1*, of *CO* (Blazquez and Weigel, 2000), demonstrating that *LFY* is a convergence point of several floral inductive signals.

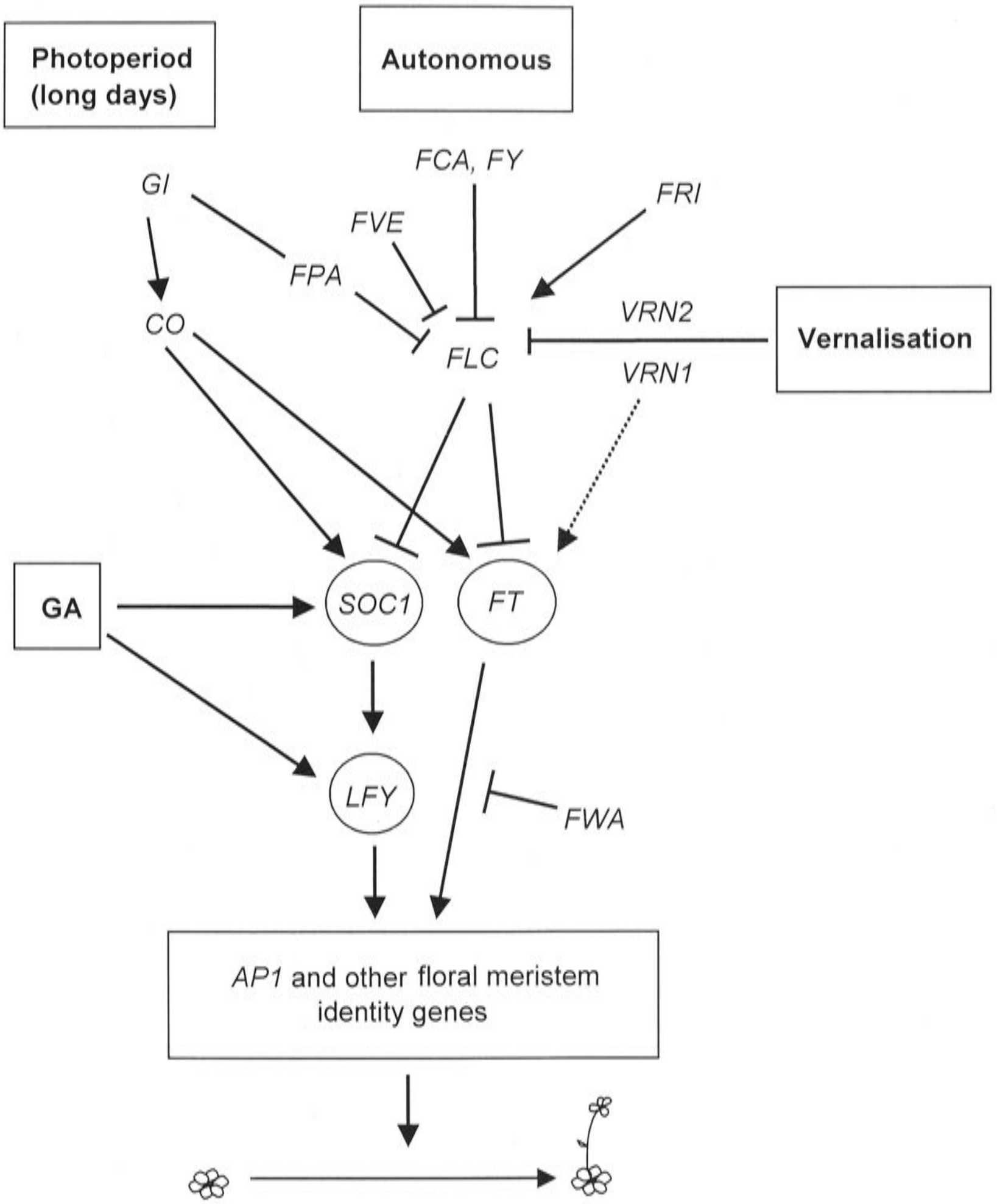


Figure 1.3. Interactions among floral promotion pathway genes in *Arabidopsis thaliana*. Integration points *LFY*, *SOC1* and *FT* are circled.

Like *LFY*, *FT* and *SOC1* are upregulated by the photoperiod pathway, via CO (Onouchi *et al.*, 2000; Samach *et al.*, 2000), but are also repressed by *FLC* (Samach *et al.*, 2000; Michaels and Amasino, 2001). The *FLC*-mediated repression of *FT* and *SOC1* is overcome by vernalisation (Lee *et al.*, 2000). *SOC1* is also up-regulated by GA (Borner *et al.*, 2000), making *SOC1* a convergence point of all four floral promotive pathways.

1.14 DNA methylation and the floral transition

If the floral promotion pathways are to function as an effective network, proper regulation of gene expression is of the utmost importance. As described in section 1.5, DNA methylation plays important roles in the regulation of gene expression and development in plants. Numerous instances of interactions between DNA methylation and components of the flowering response have been observed, indicating a role for methylation in the regulation of flowering.

A role for DNA methylation in the vernalisation response was suggested by the observation that vernalisation of tobacco cells or *Arabidopsis* results in a decrease in methylation levels (Burn *et al.*, 1993; Finnegan *et al.*, 1998a). For the vernalisation signal to be effective, it must be perceived by actively dividing tissue at the plant apex (Schwabe, 1954; Wellensiek, 1964) and is inherited through mitotic cell division, as plants flower well after their exposure to cold, i.e. after the vernalisation signal is perceived. The vernalisation signal is reset at meiosis, because the progeny of vernalised plants must also be vernalised in order to induce flowering (Lang, 1965). These properties of the vernalisation signal suggest that an epigenetic mechanism, such as DNA methylation, could act as the signal (Burn *et al.*, 1993; Dennis *et al.*, 1998).

Experiments using the chemical demethylating agent 5-azacytidine (5-azaC) have provided further supporting evidence for a role for DNA methylation in the vernalisation signal. 5-azaC is incorporated in place of cytosine during DNA replication, and is unable to be methylated; it is also thought to act by inhibiting cytosine methyltransferases, resulting in an overall decrease in methylation levels (Jones, 1985). When late flowering *Arabidopsis* ecotypes and mutants are treated with 5-azaC, some flower earlier than wild type plants. The early flowering response is limited to those ecotypes and mutants which are also responsive to vernalisation, indicating that demethylation by 5-azaC can partially substitute for a vernalisation treatment (Burn *et al.,* 1993). Further evidence of a role for methylation in the transition to flowering came from the *METI* antisense plants described in section 1.5.2. *Arabidopsis METI* antisense plants of the C24 ecotype, which have up to a 90 % reduction in CG methylation levels, flower earlier than wild type plants in the absence of a vernalisation treatment. This demonstrates that as for 5-azaC treatment, demethylation caused by reducing methyltransferase activity can also partially substitute for vernalisation (Finnegan *et al.,* 1996). However, methylation does not fully compensate for cold treatment, as vernalised wild type plants still flower earlier than *METI* antisense plants (Finnegan *et al.,* 1998a).

Just as vernalisation results in a decrease in expression of the floral repressor *FLC,* the response of *METI* antisense plants to demethylation has also been correlated with a decrease in *FLC* expression (Sheldon *et al.,* 1999). Earlier studies had proposed that vernalisation led to the demethylation and subsequent transcriptional activation of a gene promoting flowering, perhaps one involved in increasing GA biosynthesis (Burn *et al.,* 1993). The observation that *FLC* expression is decreased by demethylation, although appearing contrary to this earlier model, can be reconciled if a repressor of *FLC* is

activated by demethylation, or if changes in the methylation status of *FLC* promoter are responsible for the decrease in *FLC* expression (Sheldon *et al.*, 2000).

Although reducing methylation in plants of the C24 ecotype results in early flowering, this is not always so in other ecotypes. Introduction of the *METI* antisense construct into plants of the Col ecotype delays flowering in comparison to wild type plants (Ronemus *et al.*, 1996). A reduction in DNA methylation levels caused by a mutation in the *DDM1* gene (Vongs *et al.*, 1993) also causes late flowering in Col (Kakutani, 1997). Recently, it has become apparent that the different *FLC* and *FWA* alleles of these two ecotypes may be responsible for the opposing effects of demethylation on flowering time (Genger *et al.*, 2002). The late flowering phenotype of both the *fwa* mutant identified by Koornneef *et al.* (1991) and the *ddm1* mutant results from ectopic expression of the floral repressor *FWA*, which is normally repressed by methylation of direct repeats within the 5' region of the gene (Soppe *et al.*, 2000). As Col does not have significant levels of *FLC* expression (Sheldon *et al.*, 1999), switching off *FLC* by demethylation does not promote flowering.

Methylation has also been implicated in the regulation of genes normally involved in the formation of floral structures. In *METI* antisense plants, the floral genes *AG* and *AP3* are ectopically expressed in leaves (Finnegan *et al.*, 1996), whereas in wild type plants, these genes are expressed only in floral tissue (Weigel and Meyerowitz, 1994). The *wlc* (***w**avy **l**eaves and **c**otyledons*) early flowering mutant, in which repetitive sequences are hypomethylated, also expresses *AG* and *AP3* ectopically in leaves (Bancroft *et al.*, 1993; Hutchison and Dean, cited in Levy and Dean, 1998).

Floral abnormalities are observed in *METI* antisense plants, with phenotypes reminiscent of *superman, superman agamous, apelata1* and *apetala2* mutants (Finnegan *et al.*, 1996).

These phenotypes are thought to be associated with a dysregulation of gene expression, due perhaps to either changes in methylation status of promoter sequences or alterations in chromatin structure (Finnegan *et al.,* 1996). As described in section 1.5.5, epialleles of *superman* in mutagenised plants are densely methylated and silenced, and hypermethylation of *SUP* in the low methylation background of *METI* antisense plants (Jacobsen and Meyerowitz, 1997; Kishimoto *et al.*, 2001) leads to the *sup* mutant phenotype of extra stamens (Bowman *et al.,* 1992), demonstrating the importance of normal methylation patterns in maintaining the appropriate expression of these genes. Floral abnormalities such as reduced sepal number and unfused carpels also occur in repeatedly selfed *ddm1* mutants, where the *ddm1* mutation causes heritable lesions at unlinked loci (Kakutani *et al.,* 1996).

Several genes involved in flowering share sequence similarity with the *Drosophila* PcG genes. As described in section 1.5.2, PcG proteins establish and maintain silencing of homeotic genes throughout development via effects on higher order chromatin structure. Mutations in the *CLF* gene, which is in the same sequence family as the *Enhancer of zeste* PcG gene, cause early flowering and ectopic expression of *AG* and *AP3* in leaves (Goodrich *et al.,* 1997). *VRN2* and *EMF2*, which are also involved in flowering, show sequence similarity to the *Su(Z)12* class of PcG genes (Gendall *et al.,* 2001; Yoshida *et al.*, 2001). The similarity of these and other genes to PcG genes, combined with the involvement of chromatin remodeling genes such as *DDM1* in the floral transition, indicates that alteration of chromatin structure and DNA methylation are very important in the control of flowering (Blazquez *et al.,* 2001).

1.15 Scope of thesis

This thesis examines the role of methylation in several aspects of the transition to flowering in *Arabidopsis*. Chapter Three investigates possible roles for the methyltransferase genes *METIIa* and *METIIb*. Decreasing the expression of the *METII* genes had no detectable effect on global methylation levels but still promoted flowering, suggesting that METII might methylate highly specific sequences. Genes involved in photosynthesis were upregulated in a line with low levels of *METII* expression, suggesting that METII might regulate the expression of a gene/s upstream of photosynthetic genes.

Chapter Four analyses the opposing effects of demethylation on flowering time in two different ecotypes of *Arabidopsis*. This work showed that *ddm1*-induced demethylation could down-regulate expression of the floral repressor *FLC*. It also suggested that *FLC* expression is more likely to be down-regulated by changes in chromatin structure than by direct methylation e.g. of promoter sequences.

Chapter Five explores some of the interactions between demethylation, vernalisation and GA in a background with elevated levels of *FLC* expression. This work showed that GA and vernalisation promote flowering via separate pathways. It also suggested that some interactions might exist between demethylation and GA in the promotion of flowering.

Chapter Six draws together the general conclusions of the research presented in this thesis and discusses possibilities for future investigations.

Chapter 2: Materials and Methods

2.1 Transformation of *Arabidopsis*

Arabidopsis seeds were sown in pots in a mixture of 50:50 river sand:compost and placed at 4 °C for 2 d to break dormancy. Pots were then transferred to a 21 °C glasshouse with a light intensity of 100 μEinsteins (μE; μM m^{-2} s^{-1}) and the plants were grown for 6-8 weeks. After flowering had occurred, the bolting stems were trimmed back to encourage growth of secondary bolts, and allowed to grow for a further 4-5 d. Mature and developing siliques were trimmed from the plants before the pots were inverted and dipped into transformation solution (section 2.2) for 5 min. The pots were drained briefly and covered in plastic wrap to create a humid environment, and left at room temperature (RT) overnight before being returned to the glasshouse. Siliques were allowed to develop and were harvested approximately 3 weeks after dipping. Seed was then allowed to dry for at least one week before screening (section 2.3). The transformation protocol described is a modification of the method of Bechtold *et al.* (1993).

2.2 *Agrobacterium* solution for transformation

Agrobacterium tumefaciens bacterial stocks were stored in 80 % glycerol at -80 °C and were streaked onto LB-agar plates containing 25 μg/mL kanamycin and 50 μg/mL rifampicin when required. After 2-3 d growth at 29 °C, a generous scraping of bacteria was used to inoculate 50 mL of LB-broth. The 50 mL culture was shaken overnight at 29 °C before being added to 500 mL LB-broth, which was shaken at 29 °C for a further 36 h. A 250 mL aliquot of the culture was centrifuged for 20 min at 5000 x *g* in a Beckman JA-10

rotor at RT and the pellet was resuspended in 1 L of resuspension media containing 5 % sucrose, 0.1 mg/mL benzylaminopurine and 0.05 % Silwet "Vac-In-Stuff" wetting agent (Clough & Bent, 1998).

2.3 Screening transformed seeds

Seeds harvested from dipped plants were surface sterilised by shaking for 5 min in a solution containing 5 % sodium hypochlorite and 5 drops of Triton X-100 detergent per 100 mL, followed by 5 rinses in sterile distilled water. Sterile seeds were resuspended in 5 mL of 0.15 % Bacto-agar and sown onto large MS-agar Petri plates (Murashige and Skoog, 1962). The MS-agar plates also contained 30 μg/mL kanamycin, as the vectors used for transformation contained the selectable *NptII* marker gene, which confers resistance to the antibiotic kanamycin (pBin19; Frisch *et al.*, 1995). The plates were sealed with 3M Micropore tape and kept at 4 °C for 2 d before being transferred to a growth cabinet with a light intensity of 100 μE. Kanamycin-resistant transformed seedlings were readily identifiable after approximately 10 d. Untransformed seedlings were bleached and died after germination. Three to four week old kanamycin-resistant plants were transferred to soil, and DNA was extracted from a single leaf of each putative transformed plant for PCR analysis as per section 2.4.

2.4 PCR screening of putative transformed plants

DNA was extracted from a single leaf of putative transformed plants using a modification of the Edwards plant DNA mini-preparation (section 2.5.3) and was analysed by polymerase chain reaction (PCR) for the presence of either the selectable *NptII* gene, the *METII* 1.2 kb antisense transgene or the *METI* antisense transgene. PCR reactions typically contained

10 mM Tris pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 200 μM each of dATP, dTTP, dGTP and dCTP, 1 μM of each primer, 1 unit of Perkin-Elmer *Taq* polymerase and 1 μL of Edwards mini-preparation template DNA in a final volume of 20 μL. Primers for the *NptII* gene were GAG GCT ATT CGG CTA TGA (NPTII L) and ACT TCG CCC AAT AGC AG (NPTII R). Primers for the 1.2 kb *METII* antisense transgene were CGA TGA TTG TGT CTC TAC T (1.2AS1) and GAC GTA AGG GAT GAC GC (35SPRO). Primers for the *METI* antisense transgene were 35SPRO and CGA TCT CAG GGG TTT CCG G (ASMT01).

Cycling conditions for PCR reactions were as follows: one cycle of 94 °C 4 min, 50 °C 30 sec, 72 °C 30 sec; 34 cycles of 94 °C 10 sec, 50 °C 10 sec, 72 °C 30 sec; and a final cycle of 72 °C 10 min, 25 °C 2 min. Reaction products were resolved on 2 % agarose gels containing 50 μg/mL ethidium bromide (EtBr).

2.5 DNA extraction procedures

2.5.1 CsCl DNA extraction

The CsCl DNA extraction method was used when samples of high purity were required, e.g. for HPLC analysis. Five grams of leaf material was ground to fine powder in liquid nitrogen and transferred to 5 mL of extraction buffer containing 2 % CTAB, 100 mM Tris pH 8.0, 20 mM EDTA, 1.4 mM NaCl and 2 % β-mercaptoethanol, preheated to 65 °C. The sample was incubated at 65 °C for at least 5 min, after which 10 mL of 24:1 chloroform:isoamyl alcohol was added. The samples were shaken at RT for 15 min and centrifuged for 15 min at 1700 x *g* in a Jouan C312 benchtop centrifuge at RT. The aqueous layer was transferred to a fresh tube and the chloroform:isoamyl extraction was repeated. The aqueous layer was transferred to a 30 mL Corex tube and the DNA was

precipitated at RT overnight by the addition of 10 mL of buffer containing 1 % CTAB, 50 mM Tris pH 8.0, 10 mM EDTA and 1 % β-mercaptoethanol. The sample was then centrifuged for 10 min at 14 000 x *g* in a Beckman JS13.1 rotor at 4 °C and the supernatant was discarded. The pellet was resuspended in 2 mL of a solution containing 1 M CsCl, 50 mM Tris pH 8.0, 5 mM EDTA and 50 mM NaCl and left to resuspend for several hours. After the addition of 20 μL EtBr, the sample was overlaid onto a 2.5 mL cushion of 5.7 M CsCl solution containing 50 mM Tris pH 8.0, 5 mM EDTA and 50 mM NaCl. The gradient was centrifuged for 18 h at 36 000 rpm in a Beckman SW55 Ti rotor at 20 °C. After centrifugation, the genomic DNA band was removed using an 18 gauge needle punctured through the side of the centrifuge tube. The EtBr was extracted from the DNA sample by the addition of 3 aliquots of an equal volume of CsCl-saturated isopropanol, and the DNA was precipitated overnight at RT with 3 vol of 70 % ethanol. The DNA sample was pelleted by centrifuging for 15 min at 13 000 x *g* in a microfuge at RT and the supernatant was discarded. The pellet was resuspended in 200 μL TE buffer (10 mM Tris-Cl pH 8.0, 1 mM EDTA) and allowed to dissolve for several hours before being re-precipitated with 0.1 vol 3 M sodium acetate and 2 vol 100 % ethanol overnight at RT. The samples were centrifuged as above and the pellet was air-dried for 15 min before being resuspended in 100 μL TE buffer. DNA yield was then estimated by measuring the absorbance of the solution at 260 nm.

2.5.2 Dellaporta DNA extraction

The Dellaporta DNA extraction method was used when DNA samples of high purity were not required, e.g. for Southern analysis, and is based on the method of Dellaporta *et al.* (1983). One gram of leaf material was ground to fine powder in liquid nitrogen and transferred to 15 mL of extraction buffer containing 100 mM Tris pH 8.0, 50 mM EDTA,

500 mM NaCl and 10 mM β-mercaptoethanol. One millilitre of 20 % SDS was added and the sample was thoroughly shaken before being incubated at 65 °C for 10 min. After incubation, 5 mL of 5 M potassium acetate was added. The sample was thoroughly shaken and was then placed at 0 °C for 20 min. The sample was then centrifuged for 20 min at 20 000 x *g* in a Sorvall SA-600 rotor at 4 °C. After centrifugation, the supernatant was filtered through Miracloth (Calbiochem Corporation) into a 30 mL Corex tube containing 10 mL isopropanol. The sample was mixed thoroughly and incubated at -20 °C for 30 min before centrifugation for 15 min at 20 000 x *g* in a Sorvall SA-600 rotor at 4 °C. The supernatant was discarded and the pellet was drained for 5 min before being resuspended in 0.7 mL of a solution containing 50 mM Tris pH 8 and 10 mM EDTA. Once the pellet had resuspended, the sample was transferred to a 1.5 mL microfuge tube and centrifuged for 10 min at 13 000 x *g* in a microfuge at RT to pellet insoluble debris. The supernatant was transferred to a fresh microfuge tube and treated with 1 μL of 10 mg/mL RNAse for 10 min at RT, and was then mixed well with an equal volume of 1:1 phenol:chloroform. The samples were centrifuged for 10 min at 13 000 x *g* in a microfuge at RT, the aqueous phase was transferred to a fresh tube and the DNA was precipitated by the addition of 0.1 vol 3 M sodium acetate pH 5.2 and 0.6 vol isopropanol. The samples were then centrifuged for 10 min at 13 000 x *g* in a microfuge at RT, and the supernatant was discarded. The pellet was washed with 1 mL of 70 % ethanol and re-centrifuged as above, the supernatant was again discarded and the pellet was resuspended in 100-200 μL TE buffer. DNA yield was then estimated by measuring the absorbance of the solution at 260 nm.

A scaled-down version of the Dellaporta DNA extraction protocol was used when only 10-15 leaves were available, with all given volumes reduced by half.

2.5.3 Edwards DNA mini-preparation

DNA to be used as a template for PCR was extracted from a single leaf using a modification of the Edwards plant DNA mini-preparation (Edwards *et al.*, 1991). Leaf tissue was ground with a 1000 μL pipette tip in a 1.5 mL microfuge tube and 450 μL of extraction buffer containing 200 mM Tris pH 7.5, 250 mM NaCl, 25 mM EDTA and 0.5 % SDS was added. The sample was vortexed for 5 seconds and centrifuged at for 1 min at 13 000 x *g* in a microfuge at RT. A 300 μL aliquot of the supernatant was transferred to a fresh microfuge tube and an equal volume of isopropanol was added. The sample was mixed thoroughly and incubated at room temperature for 2-3 min. The sample was then centrifuged for a further 5 min as above and the supernatant was discarded. The pellet was air dried for 10 min and was then resuspended in 50 μL of TE buffer and stored at -20 °C.

2.6 DNA analysis

2.6.1 Genomic digests and Southern analysis

Genomic DNA (1-5 μg) was digested in 1 x universal restriction buffer (33 mM Tris acetate pH 7.9, 66 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM dithiothreitol), 2 mM spermidine, 10-20 units of restriction enzyme, and 100 μg/mL gelatin to make up a final volume of 50 μL. Digests were incubated overnight at the required temperature for each enzyme and then reduced to approximately 20 μL in a Savant Speed-vac vacuum centrifuge. The digests were then electrophoresed through 1 % agarose in 1 x TAE (Sambrook et al, 1989). After electrophoresis, the gels were stained for 15 min in 50 μg/mL EtBr. After a 5 min depurination treatment in 0.1 N hydrochloric acid, the DNA was

transferred to Hybond-N+ nylon membranes (Amersham) in 0.4 N sodium hydroxide overnight. The DNA was fixed to the membranes by exposure to UV light in a Stratagene 1800 UV-Stratalinker, the membranes were rinsed in 2 x SSC (0.15 M NaCl, 0.015M sodium citrate pH 7.0) for 2 min, wrapped in clingfilm and stored at 4 °C.

Membranes were pre-hybridised at 42 °C (low stringency) or 65 °C (high stringency) for 2-3 h in 30-40 mL of a solution containing 0.5 M sodium phosphate buffer pH 7.2, 1 % BSA, 7 % SDS and 1 mM EDTA. Radiolabelled DNA probes (labelled using α-^{32}P dCTP and the Dupont Random Primer Extension Kit) were mixed with 1 mL of 6 mg/mL salmon sperm DNA, boiled for 2 min, chilled for 2 min and then added to the membranes with 10 mL of pre-hybridisation solution. After overnight hybridisation at either 42 °C or 65 °C, membranes were washed twice at RT in 2 x SSC, 0.1 % SDS; twice at RT in 0.2 x SSC, 0.1 % SDS; and twice at 65 °C in 0.2 x SSC, 0.1 % SDS. Filters were then exposed either to x-ray film (Kodak) at –80 °C, or on Phosphorimager screens (Molecular Dynamics, Sunnyvale, CA, USA) at RT.

2.7 RNA extraction procedure

The RNA extraction protocol was based on that of Longemann *et al.* (1987). One gram of leaf material was ground to a fine powder in liquid nitrogen in a pre-cooled mortar. Two millilitres of buffer containing 8 M guanidine hydrochloride, 20 mM MES pH 7.0, 20 mM EDTA and 50 mM β-mercaptoethanol was added to the mortar and allowed to freeze. Upon thawing, the powder-buffer mix was ground further, then transferred to a 10 mL plastic centrifuge tube. Three millilitres of water-saturated phenol:chloroform:isoamyl alcohol (25:24:1) was added, the samples were shaken for 10 min at RT, and were then centrifuged for 15 min at 1700 x *g* in a Jouan C312 benchtop centrifuge at RT. The

aqueous phase was transferred to a fresh 10 mL plastic tube and the phenol:chloroform:isoamyl alcohol extraction was repeated. The aqueous phase was transferred to a 15 mL Corex tube and the nucleic acids were precipitated with 0.2 vol 1 M acetic acid and 0.7 vol 100 % ethanol at -20 °C overnight. The sample was centrifuged for 25 min at 14 000 x *g* in a Beckman JS13.1 rotor at 4 °C, and the supernatant was discarded. The pellet was dissolved in 1 mL of DEPC-H_2O, transferred to a 1.5 mL microfuge tube, and the RNA was precipitated with 220 μL of 10 M lithium chloride at 4 °C for 3-4 h. The sample was centrifuged for 15 min at 13 000 x *g* in a microfuge at 4 °C and the supernatant was discarded. The pellet was resuspended in 500 μL DEPC-H_2O and the RNA was precipitated with 0.1 vol 3 M sodium acetate and 2 vol 100 % ethanol at -20 °C overnight. The sample was centrifuged as above, the pellet was resuspended in 500 μL DEPC-H_2O and the RNA yield was estimated by measuring the absorbance of the solution at 260 nm. The RNA was precipitated as above and the pellet was resuspended in DEPC-H_2O at a final concentration of 5 μg/μL. RNA samples were stored at -20 °C for short term storage (1-2 months) or at -80 °C for long term storage.

2.8 RNA analysis

2.8.1 RNA gels and Northern analysis

Ten micrograms of total RNA was added to 20 μL sample buffer containing 65 % formamide, 20 % formaldehyde, 12.5 % 10 x MOPS buffer (0.2 M MOPS pH 7.2, 50 mM sodium acetate, 10 mM EDTA) and 0.025 % EtBr. RNA samples were made up to a final volume of 25 μL with DEPC-H_2O and were incubated at 65°C for 5 min before being electrophoresed through a 1.1% agarose gel containing 1 x MOPS and 18 % formaldehyde. Separated RNA samples were transferred to Hybond-N filters (Amersham)

overnight in 20 X SSC and were fixed by exposure to UV light in a Stratagene 1800 UV-Stratalinker. Filters were rinsed in 2 x SSC for 2 min, wrapped in clingfilm and stored at 4 °C until use.

Filters were pre-hybridised at 55 °C for 2-3 h in 10 mL of pre-hybridisation buffer (50 % formamide, 250 mM sodium phosphate pH 7.2, 250 mM NaCl, 1 mM EDTA, and 7 % SDS). Radiolabelled RNA probes (labelled using α-^{32}P dUTP and the Epicentre Technologies Riboscribe riboprobe extension kit) were added to the pre-hybridisation solution. After overnight hybridisation at 55 °C membranes were washed and exposed as per section 2.6.1. Filters requiring RNAse A treatment to remove background signals due to ribosomal trapping were washed three times in 2 x SSC for 10 min at RT, then treated with 2 μg mL^{-1} RNAse A in 2 x SSC for 15 min at RT. RNAse-treated filters were then washed for 15 min in 0.1 x SSC, 0.1 % SDS at 65 °C according to the method of Dolferus *et al.* (1994).

2.8.2 RT-PCR

Total RNA was isolated from 19 d old seedlings grown on MS plates as per section 2.3. RNA (10-100 μg) to be used as a template for RT-PCR was treated with 1-5 units of RQ1 DNase in 1 x RQ1 restriction buffer (Promega) and DEPC-treated H_2O in a final volume of 100 μL. The reaction was incubated at 37 °C for 15 min, a further 1-5 units of enzyme were added, and the reactions were incubated at 37 °C for another 15 min. The samples were then extracted with an equal volume of water-saturated phenol:chloroform, shaken thoroughly and centrifuged for 3 min at 13 000 x *g* in a microfuge at RT. The aqueous layer was transferred to a fresh microfuge tube and the RNA was precipitated with 0.1 vol 3 M sodium acetate and 2 vol 100 % ethanol overnight at -20 °C. The samples were

centrifuged for 15 min at 13 000 x *g* in a microfuge at 4 °C, the supernatant was carefully aspirated and the pellet was resuspended in 20-40 μL of DEPC-H_2O.

RT-PCR was performed using the Access RT-PCR system (Promega). RT-PCR reactions contained 1 x AMVRT/Tfl buffer, 2.5 mM $MgSO_4$, 0.2 mM dNTP's, 50 ng each primer, 2 units of AMV reverse transcriptase, 2 units of *Tfl* polymerase and 1 μg of DNase-treated RNA in a final volume of 20 μL. Primers were designed to span introns, thus providing a size differential between products amplified from contaminating genomic DNA and from cDNA.

2.9 Flowering time experiments

Seeds to be used for flowering time experiments were surface sterilised as per section 2.3 and sown either in individual sterilised test tubes containing 7 mL of MS-noble agar (pH 7.0) with 1 x MS iron, macro- and micro-nutrients, 0.2 x MS vitamins (Murashige and Skoog, 1962) and 1.5 % sucrose, or on large Petri plates containing approximately 100 mL of MS-noble agar as above. The seeds were incubated at 4 °C for 2 d to break dormancy and were then transferred to a growth cabinet kept at 21 °C. Seeds which were to be vernalised were incubated in darkness at 4 °C for 3 weeks before being transferred to the same growth cabinet. Seeds which were to be treated with gibberellic acid (GA) had a final concentration of 10^{-4} M GA_3 added to the growth media before the tubes or plates were poured.

Racks and plates were moved daily to ensure that all plants received an even amount of light. Plants were observed daily and flowering time was recorded as the number of days from germination to visible elongation of the bolting stem. The number of rosette leaves

present when bolting occurred and the total number of leaves (rosette + cauline leaves) at flowering was also recorded, as a good correlation exists between flowering time and leaf number (Koornneef *et al.*, 1991). The Mann-Whitney U-test was used to determine statistical significance of observed differences in flowering time (Snedecor and Cochran, 1967).

2.10 DNA sequencing

Sequencing was carried out using the Perkin-Elmer "Big Dye" dye terminator sequencing kit. PCR reaction products were purified using Qiaquick PCR minicolumns (Qiagen) and resuspended in 30 μL TE. Purified template DNA (50-100 ng) was then added to 4 μL Big Dye mix and 1 μM primer in a final volume of 10 μL. Cycling conditions for the PCR were as follows: one cycle of 95 °C 2 min; 25 cycles of 95 °C 20 sec, 50 °C 15 sec, 60 °C 4 min; and a final cycle of 25 °C 1 min. PCR products were precipitated with 5 vol 100 % ethanol and 0.02 vol 3 M sodium acetate pH 5.2 for 15 min at -20 °C, then centrifuged for 15 min at 13 000 x *g* in a microfuge at 4°C. The supernatant was discarded by aspiration and the pellet washed with 500 μL 70 % ethanol, vortexed and recentrifuged as above. The supernatant was again aspirated and the pellet was dried in a vacuum centrifuge for 5 min. Sequencing was performed using an Applied Biosystems model 370A DNA sequencer and sequence data was analysed using GCG software (The Wisconsin Sequence Analysis Package, Genetics Computer Group).

Chapter 3: A role for the methyltransferase METII in *Arabidopsis*

3.1 Introduction

Plant DNA methyltransferases have been identified in many plant species, from pea and carrot to maize and tomato (Bernacchia *et al.*, 1998a, 1998b; Pradhan *et al.*, 1998; Papa *et al.*, 2001). The crucifer *Arabidopsis thaliana* has at least 10 methyltransferase genes (The *Arabidopsis* Genome Initiative, 2000) which fall into at least three classes, known as the *METI*, *CMT* and *DRM* classes (Finnegan and Dennis, 1993; Henikoff and Comai, 1998; Cao *et al.*, 2000).

The *Arabidopsis METHYLTRANSFERASE1 (METI)* class has four members; *METI, METIIa, METIIb* and *METIII* (Genger *et al.*, 1999). Gene structure, including the position of all 11 introns, is conserved between the members of the *METI* class and they share a high degree of sequence identity, especially within the methyltransferase domain (Finnegan and Kovac, 2000). *METI*, which was the first plant methyltransferase gene to be identified, was isolated on the basis of similarity to the mouse methyltransferase *Dnmt1* (Finnegan and Dennis, 1993). *METI* is the most highly expressed member of this class and is expressed in all tissues. *METIIa* and *METIIb*, which share up to 80% identity with *METI* (Finnegan and Kovac, 2000) are also ubiquitously expressed, but at levels of about 10,000-fold lower than *METI* in most tissues (Genger *et al.*, 1999). *METIIa* and *METIIb* are most similar to the *METI* class of genes, and are more than 90% identical to each other over their entire length (Genger *et al.*, 1999). No expression studies have been reported

for *METIII*, which is about 80% identical to the *METII* genes (Finnegan and Kovac, 2000). In the Columbia ecotype, *METIII* encodes a truncated non-functional protein (Genger *et al.,* 1999) but in C24 it encodes an intact reading frame, suggesting that it may be functional in this and possibly other ecotypes (EJ Finnegan, personal communication). To date, no functions have been attributed to *METIIa, METIIb* or *METIII* (Finnegan and Kovac, 2000).

Like its mouse counterpart *Dnmt1*, *METI* acts principally as a maintenance methyltransferase and preferentially methylates cytosines within CG dinucleotides in both single copy and repeated DNA sequences (Finnegan *et al.,* 1996; Ronemus *et al.,* 1996; Kishimoto *et al.*, 2001). As DNA methylation is found not only within CG sequences but also within CNG and asymmetric sequences (Bender and Fink, 1995; Oakeley and Jost, 1996), *METI* cannot be the sole methyltransferase responsible for cytosine methylation in *Arabidopsis,* suggesting roles for other methyltransferases.

The *CHROMOMETHYLTRANSFERASE (CMT)* class of methyltransferases, which is specific to plants, is distinguished from the *METI* class by the presence of a chromodomain within the methyltransferase domain (Henikoff and Comai, 1998). CMT enzymes methylate CNG, asymmetric, and to a lesser extent, CG sequences, in regions of heterochromatin (Henikoff and Comai, 1998; Bartee *et al.,* 2001; Lindroth *et al.,* 2001). The third class of methyltransferase, the *DOMAINS REARRANGED METHYLTRANSFERASE (DRM)* class, differs from both the *METI* and the *CMT* class in the arrangement of the conserved amino acid motifs. The *DRM* class shares similarities with the *Dnmt3* class of *de novo* methyltransferases of mice and humans (Cao *et al.,* 2000).

DNA methylation is important in many aspects of plant development. *METI* insertion mutants have recently revealed that METI maintains CG methylation during gametogenesis (Saze *et al.,* 2003). Plants expressing an antisense transgene against the methyltransferase *METI* (AMT; anti-methyltransferase) have a pleiotropic phenotype, with features including reduced apical dominance, altered leaf size and shape, decreased fertility and ectopic expression of floral homeotic genes in leaf tissue (Finnegan *et al.,* 1996; Ronemus *et al.,* 1996). Floral abnormalities caused by homeotic transformation of floral organs are also observed in AMT plants (Finnegan *et al.,* 1996). These latter phenotypes are associated with local regions of hypermethylation and transcriptional silencing (Jacobsen and Meyerowitz, 1997; Jacobsen *et al.,* 2000). As the activity of METI is compromised in these plants, and because METI is thought to be a maintenance methyltransferase, this *de novo* local hypermethylation is attributed to the action of other methyltransferases; potential candidates are the *Dnmt3*-like *DRM* class of methyltransferases (Cao *et al.,* 2000). Further evidence for roles of methyltransferases other than METI in plant development is provided by the fact that even the most severely demethylated AMT lines still have around 10 % of the wild type levels of CG methylation (Finnegan *et al.,* 1996).

Decreasing methylation levels in plants can affect the time taken for flowering to occur. In the C24 background, AMT plants flower earlier than wild type plants (Finnegan *et al.,* 1998). Flowering in *Arabidopsis* is also promoted by vernalisation (Napp-Zinn, 1985). The early flowering caused by both AMT and vernalisation is associated with a reduction in expression of the flowering repressor *FLC* (Sheldon *et al.,* 1999). A four-week vernalisation treatment reduces methylation levels of wild type *Arabidopsis* plants by about 15% compared to unvernalised plants (Finnegan *et al.,* 1998). AMT plants that flowered at the same time as vernalised wild type plants had a 70% reduction in methylation,

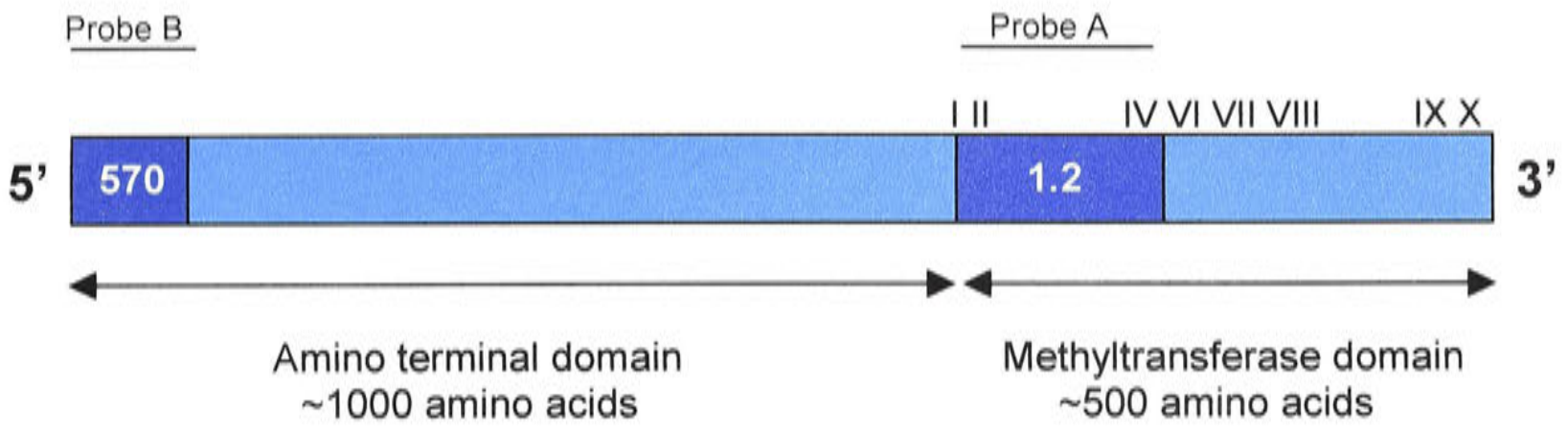
indicating that the methylation status of some specific sequences might be important for the early flowering response to vernalisation (Finnegan *et al.*, 1998). Demethylation in AMT plants decreased but did not prevent a vernalisation response, as they flowered earlier still when vernalised (Finnegan *et al.*, 1998), suggesting that perhaps certain sites important for the vernalisation response are methylated by an enzyme other than METI.

The experiments reported in this chapter aimed to investigate the role of the methyltransferase gene formerly known as *METII* (which after the discovery of the *METIIb* gene, was renamed *METIIa;* Genger *et al.*, 1999) in the growth and development of *Arabidopsis* and to determine if like *METI, METII* plays a role in the transition to flowering. To enable this investigation, transgenic plants with reduced levels of *METII* expression were generated and characterised.

3.2 Materials and methods

3.2.1 Construct design and transformation

Two constructs were used to transform *Arabidopsis thaliana* ecotype C24 (Figure 3.1). Construct A (gift of R. Genger) contained an antisense 1.2 kb *Hin*dIII fragment of genomic DNA spanning motifs I to IV of the methyltransferase domain of *METII*. Construct B contained a sense 570 bp *Bam*HI fragment of genomic DNA from the 5' end of the amino-terminal domain of *METII*, including 10 bp of 5' untranslated region and 560 bp of coding sequence. The sense construct was used with the aim of silencing *METII* by cosuppression, which can occur in lines with multiple copies of a sense transgene (Napoli *et al.*, 1990). The *METII* fragments were initially cloned into the pJ35SN plasmid (Finnegan *et al.*, 1996) which contains the constitutive cauliflower mosaic virus 35S promoter. These



(A)

(B)

Figure 3.1 (A), Structure of the *METII* gene. The regions of the 570 bp sense and 1.2 kb antisense fragments are shaded dark blue. Positions of conserved amino acid motifs are shown in Roman numerals. Regions used as probes in Southern analyses are indicated by lines above the figure. **(B)**, Constructs used for transformation of *Arabidopsis*. The 1.2 kb antisense fragment of *METII* was cloned into pJ35SN as a *Hin*dIII fragment. The 570 bp sense fragment of *METII* was cloned into pJ35SN as a *Bam*HI fragment.

plasmids were then cloned separately into the pBin19 plasmid which contains the *NptII* kanamycin resistance selectable marker gene (Figure 3.1; Frisch *et al.*, 1995). The pBin19 plasmids were then introduced into *Agrobacterium tumefaciens* strain AGL-1 in a triparental mating using the helper strain RK2013.

The two constructs were transformed into C24 as per sections 2.1 and 2.2. Transformed plants were identified by screening T1 seeds on MS medium containing kanamycin as per section 2.3. Kanamycin-resistant plants were scored for the presence of the 1.2 kb *METII* antisense transgene (for plants transformed with construct A) or the *NptII* selectable marker gene (for plants transformed with construct B) by PCR as per section 2.4.

3.2.2 Analysis of *METII* expression level

3.2.2.1 By RT-PCR

T2 plants were screened using reverse-transcriptase polymerase chain reaction (RT-PCR) as per section 2.8.2 to ascertain whether the level of *METII* expression was affected. Primers for *METI* were GAA TTC CAG CCC ATG GGT AAG (Met1MIX5') and GAA TTC GGG TTG GTG TTG AGG (Met1MIX3'); primers for *METII* were CTT CAT ATT CGC CGG AAA CTG C (RTXII) and AGC TAG TAA GGC TTC ATT TCA G (NEWI). Cycling conditions for the *METI* RT-PCR were as follows: one cycle of 48 °C 45 min, 95 °C 2 min; one cycle of 95 °C 2 min, 44 °C 30 sec, 71 °C 1 min; nine cycles of 93 °C 10 sec, 44 °C 10 sec, 71 °C 30 sec; 25 cycles of 93 °C 10 sec, 60 °C 10 sec, 71 °C 30 sec; and a final cycle of 93 °C 10 sec, 60 °C 10 sec, 71 °C 5 min, 25 °C 2 min. Cycling conditions for the *METII* RT-PCR were as follows: one cycle of 48 °C 45 min, 94 °C 2 min; 35 cycles of 94 °C 30 sec, 58 °C 1 min, 68 °C 30 sec; and a final cycle of 68 °C 7 min, 25 °C 2 min.

Reaction products were electrophoresed on 2 % agarose gels, transferred to Hybond-N+ membranes (Amersham) and hybridised as per section 2.6.1. to either the *METII* 1.2 kb probe A (Figure 3.1) or the *METI* Y8 cDNA probe. The *METI* probe pBGSY8 consisted of a 2.8 kb cDNA covering the 3' untranslated region, 1.5 kb of the methyltransferase domain and 1.2 kb of the amino terminal domain of *METI*. Filters were exposed on Phosphorimager screens as per section 2.6.1. Signals were quantified using Imagequant software version 3.3. The ratio of *METI:METII* signal intensities in triplicate samples of each transgenic line was normalised to the ratio of *METI:METII* signal intensities in the C24 control line. Transgenic lines that appeared to have reduced levels of *METII* expression as determined by RT-PCR were analysed further using real time PCR.

3.2.2.2 By real time PCR

For real time PCR, RNA was isolated from 19 d old seedlings grown on MS plates as per section 2.3 and 100 μg of total RNA was DNAse treated as per section 2.8.2. First strand cDNA synthesis was carried out using the Gibco BRL Superscript II kit. One microgram of oligo-dT primer was annealed to the DNAse treated RNA at 70 °C for 10 min. The primer-RNA mix was then added to reverse transcription reaction mix, which contained 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM dNTPs and 0.2 units of Superscript II RNA polymerase. The reverse transcription reaction was incubated for 1 h at 42 °C. The reaction product was purified using a Qiaquick minicolumn to remove excess dNTPs and was resuspended in 30 μL TE buffer.

PCR reactions were performed using a Corbett Research Rotor-gene 200 real time cycler and the results were analysed using Rotor-gene software version 4.3. A standard curve was generated for each experiment using 10-fold serial dilutions (from 5,000 fg/μL to

0.05 fg/μL) of the *METI* PCR product amplified from C24 cDNA. The standard curve was calculated by the Rotor-gene software using cycle threshold (CT) values. The PCR product crosses the CT when the fluorescence from incorporated SYBR-Green reaches more than 10 standard deviations above the background fluorescence level. The time at which the product crosses the CT depends of the concentration of the starting template; therefore, the CT value is a reliable indicator of the initial copy number of a particular template. Two-fold dilutions of each cDNA sample were used to ensure that the reaction was dose-dependent, as the CT is reached one cycle earlier when the amount of starting template is doubled. The dilutions used were as follows: for *METI* reactions, 80 and 40 ng/5μl; for *METII* reactions, 200 and 100 ng/5μL. The amounts chosen reflected the amount needed for a reliable, reproducible PCR product, the cycle threshold of which fell between the two extremes of the standard curve. Real-time PCR reactions contained 20 mM Tris-HCl pH 8.4, 50 mM KCl, 3.5 mM $MgCl_2$, 0.2 mM dNTPs, 50 ng of each primer, 1 μL of a 1:2500 dilution of SYBR-Green fluorescent dye (Molecular Probes), 0.5 units of Platinum *Taq* polymerase (Life Technologies) and 5 μL of the appropriate cDNA dilution in a final volume of 20 μL. Primers used for *METI* were Met1MIX5' and Met1MIX3' (section 3.2.2.1). Primers for *METII* were ATC CAA GAT AAA CAA AGA GTA TGG AAC (METIIL) and CCA CGA GTC CAT TTG ATA ATG TC (METIIR). Cycling conditions were as follows; one cycle of 94 °C 2 min, then 45 cycles of 94 °C 30 sec, 60 °C 30 sec, 72 °C 30 sec. A melt curve was then performed as follows; 50 °C 1 min, 55 °C 1 min, followed by 55 °C to 99 °C in one degree increments, held for 5 sec per increment.

The ratio of *METII:METI* expression was calculated by the Rotor-gene software using values obtained from the standard curve, measured in femtograms of starting material.

3.2.3 Characterisation of transgenic lines: copy number, segregation and progeny testing

Southern analysis for copy number and segregation was carried out as per section 2.6.1 using DNA from five individual soil-grown T2 plants and a pooled sample of the same plants. For progeny testing, 100-150 T2 seedlings were grown on MS plates containing 50 μg/mL kanamycin as per section 2.3. The number of resistant and sensitive seedlings was recorded after 3 weeks growth. The light intensity in the growth cabinet used for kanamycin selection was approximately 50-60 μE.

3.2.4 Analysis of methylation levels

Total genomic levels of methyl cytosine (^{m}C) were determined using reversed-phase high performance liquid chromatography (RP-HPLC). DNA digests for RP-HPLC analysis were based on the method of Gehrke *et al.* (1984) and included 5 μg DNA, 15 mM sodium acetate pH 5.3, 0.5 mM zinc sulphate, 3 μg P1 nuclease (Boehringer Mannheim) and 0.4 units thermosensitive alkaline phosphatase (TsAP; Life Technologies) made up to final volume of 150 μL in Milli-Q deionised water. Digests were incubated at 37 °C for 2 h, then 15 μL of 0.5 M Tris pH 8.5 was added to activate the TsAP. The digests were incubated for at least another 2 h at 37 °C and were stored at -20 °C until use.

Digested DNA was separated on a Supelco Supelcosil LC-18-S HPLC column (15 cm x 4.6 mm x 5 μm) at a flow rate of 1.0 mL/min. The column was equilibrated at 35 °C with a buffer solution containing 50 mM potassium dihydrogen phosphate pH 4.0 and 2.5 % redistilled methanol. The buffer solution was filtered through a 0.22 μm Millipore GVWP filter before use. A Waters RP-HPLC system equipped with a fixed wavelength UV

detector set at 254 nm was used to detect the eluates. DNA and RNA standards were run on the column separately to ascertain the correct conditions and retention times for each standard. An aliquot of the standards mixture was injected into the column before each run to correct for any minor deviations in retention time due to differences in batches of buffer. The mol % of base composition was calculated by standardisation of each peak area with respect to the standard bases. The amount of ^{m}C in each sample was calculated using the formula ^{m}dC/(^{m}dC+dC).

Methylation levels at specific sites were assayed by the use of methylation-sensitive restriction enzymes. Genomic DNA was digested overnight at 37 °C as per section 2.6.1 with either *Msp*I or *Hpa*II to assay methylation at CCGG sites, or with *Pvu*II to assay methylation at CNG sites. Filters were hybridised to either a 180 bp centromeric repeat probe (for *Msp*I/*Hpa*II digests) or a 28S ribosomal repeat probe (for *Pvu*II digests) at 65 °C as per section 2.6.1.

3.2.5 Flowering time experiments

Analysis of flowering time, vernalisation response and GA response was carried out as per section 2.9. Wooden racks containing 20 tubes were placed under long day (LD) conditions (16 h light, 8 h dark) with a light intensity of 125-175 μE. Flowering time experiments were also carried out on 140 mm diameter Petri plates containing MS medium as per section 2.9. The light intensity for experiments using Petri plates was 120-130 μE.

3.2.6 Analysis of *FLC* and *SOC1* expression

Northern analysis of total RNA was carried out as per section 2.8.1. The *FLC* riboprobe was prepared from the pZLMADS plasmid. The plasmid was linearized with *Sty*I to produce a probe containing the complete *FLC* cDNA excluding the MADS box region (Sheldon *et al.*, 1999). The *SOC1 (AGL20)* riboprobe was prepared from the *Sal*I-linearised EST 179H16 plasmid which contained 590 nucleotides of *SOC1* 3' terminal sequence but lacked the MADS-box coding region (Sheldon *et al.*, 1999).

3.2.7 Analysis of *fis* phenotype rescue

Pollen from *METII* transgenic lines was used to pollinate emasculated, heterozygous *Arabidopsis* mutants *fis1* and *fis2*. Wild type C24 and *METI* antisense plants were crossed to the *fis* mutants as negative and positive controls, respectively. Siliques were dissected 10-15 d after crossing and seed phenotype was examined using a dissecting microscope.

3.2.8 Microarray analysis

Plants for microarray experiments were grown on 140 mm diameter MS-agar Petri plates as per section 2.9 for 19 d in LD conditions with a light intensity of 85-95 μE. Four biological replicates were used in the analysis. Pairs of plates to be compared were placed next to each other under the lights and all growing and harvesting conditions were kept as similar as possible. RNA was extracted as per section 2.7 and cDNA was prepared from 100 μg of total RNA as per section 3.2.2.2.

cDNA products were treated with 1.0 unit of RNAse H for 30 min at 37 °C to destroy RNA-DNA hybrids, purified using Amicon Microcon YM30 filters (Millipore) and dried under

vacuum for 10 min at 42 °C. The purified cDNA was then labelled with either Cy-3 or Cy-5-labelled dUTP (Amersham Pharmacia Biotech) by a randomly primed polymerase reaction. Reactions contained 25 ng of cDNA, 1x Klenow fill-in buffer (USB), 3 μg random hexanucleotide primers (Gibco), 25 nmol of either Cy-5 dUTP or Cy-3 dUTP, 0.25 mM of dNTPs (A, C and G), 0.09 mM dTTP and 5 units of Klenow polymerase in a final volume of 20 μL. The cDNA, buffer and primer mix was incubated for 2 min at 99 °C and allowed to cool for 5 min at RT before addition of the remaining components. The reactions were incubated for 3 h at 37 °C. Each cDNA sample was labelled separately with Cy-5 and Cy-3, with the combination of fluors being reversed for two of the four replicates to allow for any effects caused by differential incorporation of the two labels. Labelled Cy-5 and Cy-3 cDNAs for each pair of samples to be compared were combined, purified using Amicon filters and dried as above. The labelled probes were resuspended in 30 μL of hybridisation buffer containing 50 % formamide, 5x SSC, 0.1 % SDS and 30 μg of salmon sperm DNA, incubated for 3 min at 95 °C, then cooled on ice.

CMT-GAPS coated slides (Corning Microarray Technology) from Mendel Biotechnology (Hayward, CA) printed with a total of 13,680 spots were used for the microarray analysis. Of these, 7680 spots represented 7212 unique clones of *Arabidopsis thaliana* cDNAs (Helliwell *et al.*, 2001). The slides were supplemented with ~6000 additional *Arabidopsis* clones (Iain Wilson, personal communication) Slides were pre-hybridised in 50 % formamide, 5x SSC, 0.1 % SDS and 1 % BSA for 1 h at 42 °C, rinsed in distilled water for 2 min at RT, and dried in a Sigma 4K15C benchtop centrifuge for 2 min at 1000 rpm. The probe was pipetted onto the slide and a coverslip was added. The slides were placed in waterproof hybridisation chambers and incubated in a 42 °C water bath overnight. After hybridisation, slides were gently washed in 2 x SSC at 42 °C to remove the coverslip, and

were then washed twice with shaking in 0.1 x SSC, 0.1 % SDS for 5 min at RT and twice in 0.1 x SSC for 5 min at RT before being dried as above.

Slides were scanned using a GenePix 4000A Microarray Scanner (Axon Instruments) and analysed using GenePix Pro 3 software. The software uses predefined grids which were manually adjusted to ensure optimal spot recognition. Spots with dust, high local background or other abnormalities were flagged as bad and ignored in subsequent analyses. The microarray data were analysed using the tRMA program version 1.6.2 (Wilson *et al.*, 2003). The data were spatially normalised and the median fluorescence values of four replicate slides were averaged. The median ratio of red:green fluorescence was then normalised, with a value of 1.0 representing no change in expression between samples. Genes with a normalised red:green ratio of more than 2 (a 2-fold increase) or less than 0.5 (a 2-fold decrease) on at least three of the four replicate slides were classified as differentially expressed (Helliwell *et al.*, 2001).

3.3 Results

3.3.1 *METII* is part of a multigene family

The copy number of the *METII* gene in the *Arabidopsis* genome was examined by high stringency Southern blot analysis of C24 and Columbia (Col) DNA digested with *Hin*dIII and *Eco*RI, using two *METII* probes (Figure 3.1; Probe A and Probe B). Probe A was a 1.2 kb fragment spanning motifs I to IV of the methyltransferase domain, a region which has 79 % identity to *METI*. Probe B corresponded to 570 bp of the 5' amino terminal domain of *METII*, where homology to *METI* decreases to 68 % (Genger *et al.*, 1999).

Probes A and B both hybridised to multiple fragments in genomic DNA of C24 and Col (Figure 3.2a). In the initial phases of the work reported here, only one *METII* gene was known, but subsequent database searches revealed two members of the *METI* family that were more similar to *METII* than to *METI* (Genger *et al.*, 1999). One of these genes was shown to be more than 90 % identical to *METII* – this gene (accession #AC005359) was subsequently named *METIIb* and *METII* (accession #AF138283) was renamed *METIIa*. The other gene was named *METIII* (accession #AL049656). When regions of *METI* corresponding to probes A and B were hybridised to C24 and Col DNA, a single band was seen (Figure 3.2a, shows 5' *METI* probe only). There was no cross-hybridisation between *METI* and the other three members of this gene family at high stringency, indicating that these regions of *METI* are unique in the *Arabidopsis* genome (Genger *et al.*, 1999).

The Southern hybridisation data fit well with the hybridisation patterns predicted from the Col genomic sequence available in the database. Scanning the sequence for *Hin*dIII sites in the *METIIa, METIIb* and *METIII Arabidopsis* sequences available in the database indicated that the 1.2 kb *METII* probe A would hybridise to fragments of 1.0-1.2 kb in length (Figure 3.2b). There was only one band in the Col *Hin*dIII digested lane (Figure 3.2a), but as it is fairly diffuse, it may contain all three fragments. The 5' *METII* probe B hybridises to three fragments in the Col *Hin*dIII digest (Figure 3.2a), corresponding to the 2.1, 2.8 and 3.6 kb bands as predicted from the database sequence (Figure 3.2b). The 5' *METI* probe hybridised to a single band of approximately 4.3 kb in the Col *Hin*dIII digest, as predicted (Figures 3.2a, 3.2b) and a single band was also seen when a *METI* probe corresponding to the region of probe A was used (result not shown). RFLP's between the Col and C24 sequences resulted in the *METII* probe A hybridising to two fragments in the C24 *Hin*dIII digests. The RFLP must be in either *METIIb* or *METIII*, as there is no *Hin*dIII site in this region of the C24 *METIIa* sequence (R Genger, personal communication).

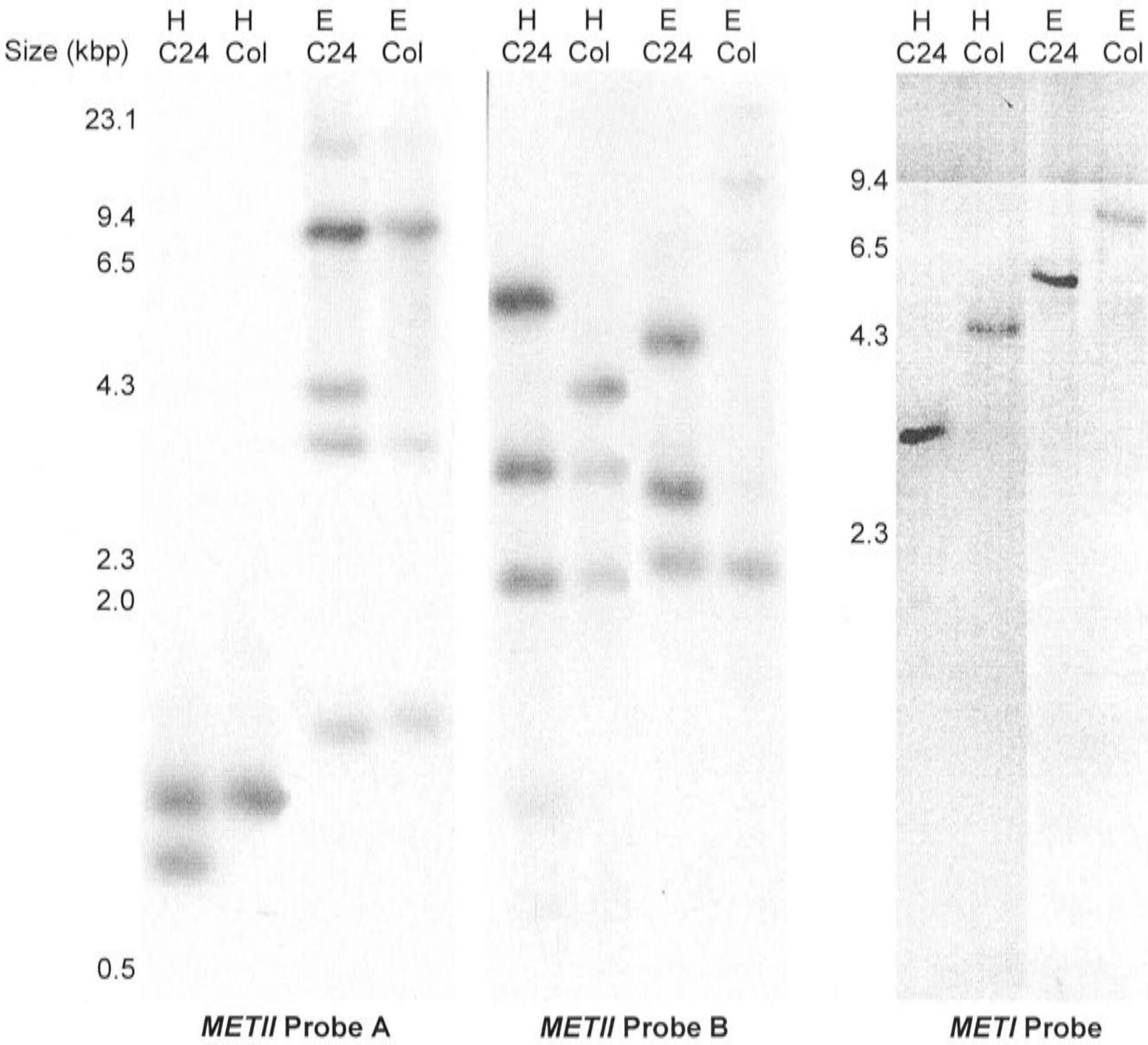
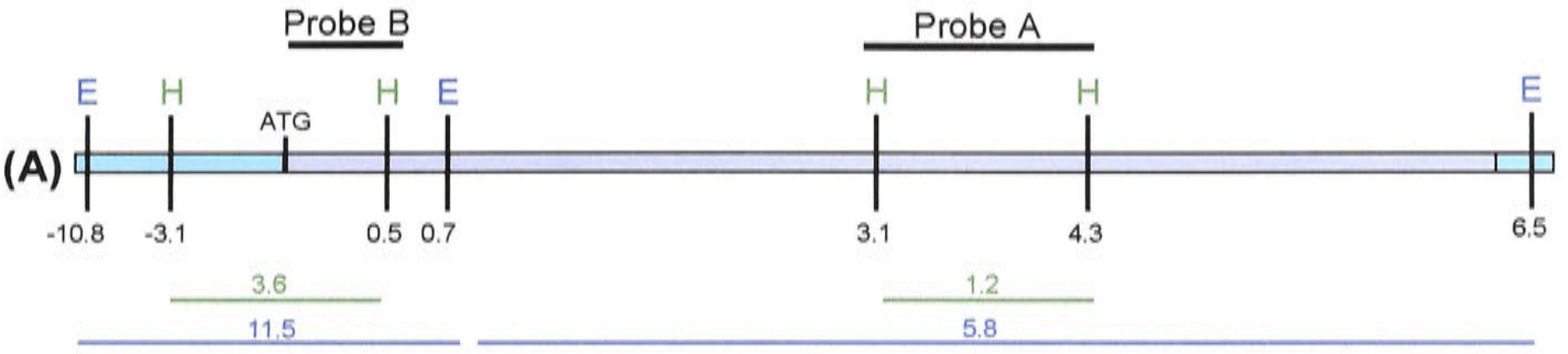
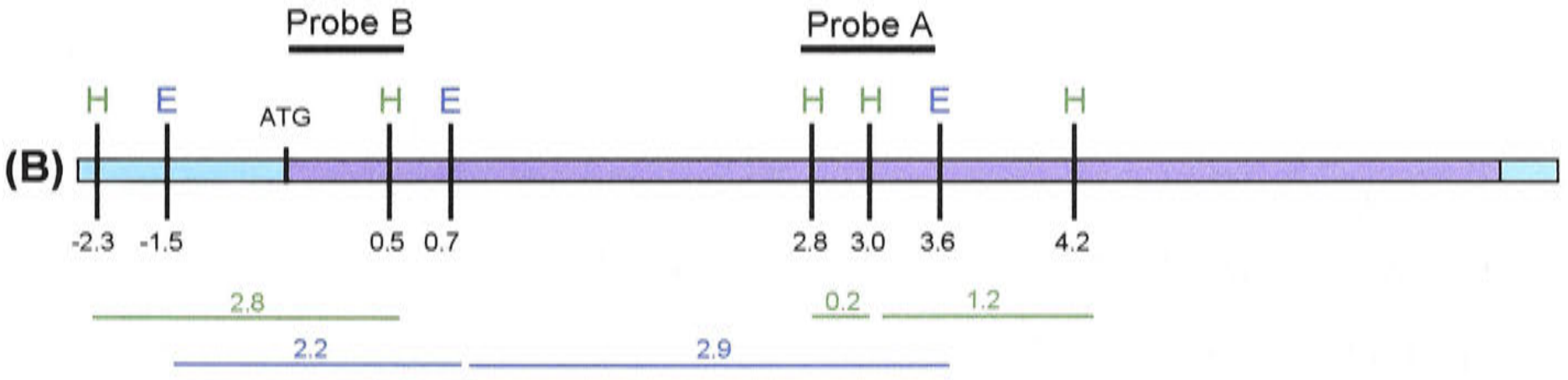
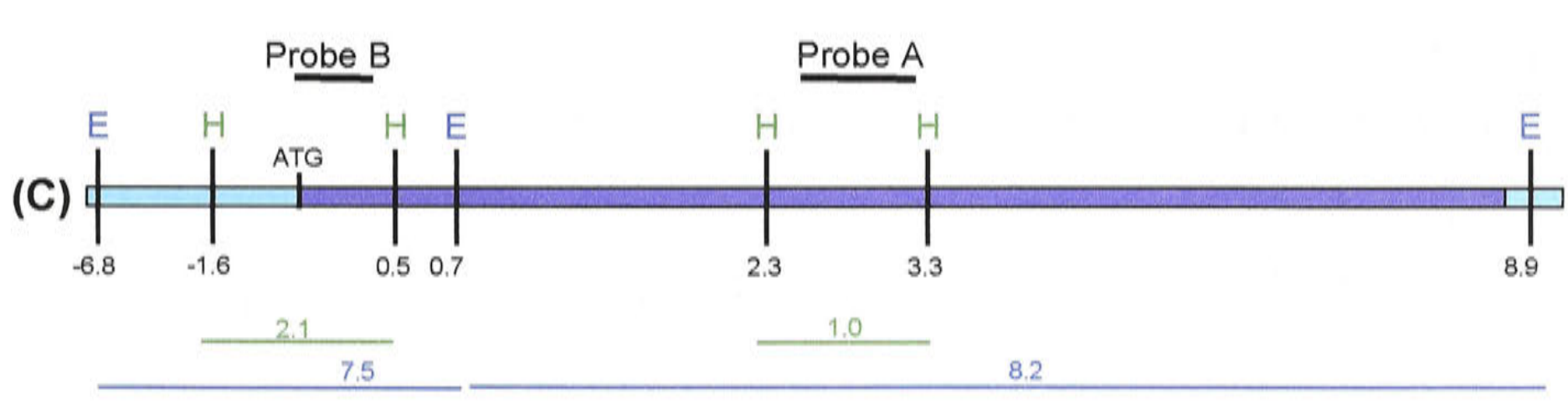


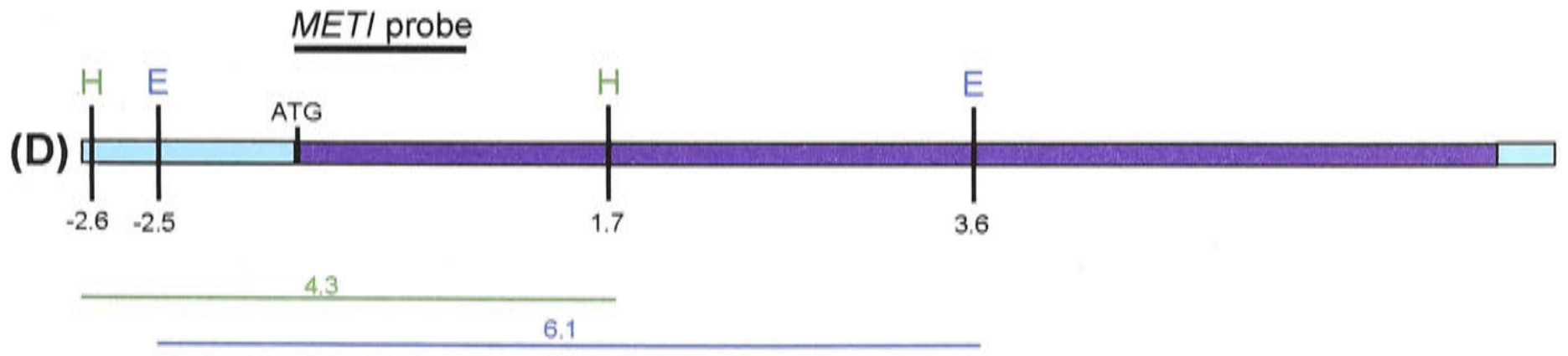
Figure 3.2a Southern analysis of the *METII* and *METI* genes in C24 and Columbia (Col) genomic DNA digested with *Hin*dIII (H) or *Eco*RI (E) and separated on 1% agarose gels. DNA was hybridized to either the *METII* 1.2 kb probe A (Figure 3.2b), the *METII* 570 bp probe B (Figure 3.2b), or Y2-4, a ca. 700 bp 5' *Bam*HI fragment of *METI* (Figure 3.2b). Multiple copies of both regions of *METII* were detected using these probes, but the *METI* probe hybridized to a single fragment. Similar results were obtained using a *METI* probe that corresponded to the 1.2 kb region of *METII* (results not shown).

Figure 3.2b *Hin*dIII and *Eco*RI sites in the four genes of the *METI* class of methyltransferases. **(A)**, *METIIa;* **(B)**, *METIIb;* **(C)**, *METIII;* **(D)**, *METI.*
The expected fragment size, based on the Columbia sequence, is given underneath in kb. Green lines = *Hin*dIII digests, blue lines = *Eco*RI digests. Upstream and downstream regions, highlighted in light blue, are not drawn to scale. For *METIIb* and *METIII*, regions of homology to the *METIIa* probes are indicated by thick black lines above each gene. The regions of homology corresponding to the *METIIa* probes are not necessarily the same size in *METIIb* and *METIII.*


Probe B
Probe A
(A)
E
H
ATG
H
E
H
H
E
-10.8
-3.1
0.5
0.7
3.1
4.3
6.5
3.6
1.2
11.5
5.8


Probe B
Probe A
(B)
H
E
ATG
H
E
H
H
E
H
-2.3
-1.5
0.5
0.7
2.8
3.0
3.6
4.2
2.8
0.2
1.2
2.2
2.9


Probe B
Probe A
(C)
E
H
ATG
H
E
H
H
E
-6.8
-1.6
0.5
0.7
2.3
3.3
8.9
2.1
1.0
7.5
8.2


METI probe
(D)
H
E
ATG
H
E
-2.6
-2.5
1.7
3.6
4.3
6.1

Probe B still hybridised to three fragments in C24 but one of them was larger than the corresponding Col fragment (Figure 3.2a).

Scanning the same sequences for *Eco*RI sites indicated that probe A would hybridise to fragments of 2.9, 5.8 and 8.2 kb (Figure 3.2b). Although three hybridising fragments were observed on the Southern (Figure 3.2a), only the 8.2 kb fragment of *METIII* corresponds to the expected size; the other two fragments are smaller than predicted. Probe B was predicted to hybridise to fragments of 2.2, 7.5 and 11.5 kb (Figure 3.2b); the largest of these is just visible and the smallest is clearly observed in the Col *Eco*RI digest (Figure 3.2a), but the predicted 7.5 kb fragment of *METIII* is not visible. As the strain of Col used for the Southern hybridisation was different to the one used for the database sequence, there could be sequence polymorphisms between the two strains that explain these inconsistencies. RFLP's between the Col and C24 sequences result in probe A hybridising to four fragments in the C24 *Eco*RI digests, with a fifth band (~20 kb) in the C24 *Eco*RI lane likely to represent incompletely digested DNA. Probe B hybridised to three fragments in C24 *Eco*RI-digested DNA.

3.3.2 Identification of *METII* transgenic lines

To investigate the role of *METII*, *Arabidopsis* was transformed with the two constructs previously described (section 3.2.1) to observe the effect of a reduction in *METII* expression. The constructs contained the *NptII* selectable marker (Figure 3.1) which confers resistance to the antibiotic kanamycin. Therefore, seed from transformed *Arabidopsis* plants of the C24 ecotype was initially screened on media containing kanamycin. Thirty-four independent kanamycin-resistant transgenic plants were identified (Table 3.1). Of these transformants, 29 contained the 1.2 kb antisense fragment (construct

Line Number	Construct	PCR	Expression reduced?[a]
1.44	A	1.2AS1, 35SPRO	-
3.1	A	1.2AS1, 35SPRO	-
3.2	A	1.2AS1, 35SPRO	-
6.1	A	1.2AS1, 35SPRO	-
6.2	A	1.2AS1, 35SPRO	Y
6.3	A	1.2AS1, 35SPRO	-
7.1	B	NptII L, NptIIR	-
7.2	B	NptII L, NptIIR	Y
8.1	A	1.2AS1, 35SPRO	-
9.1	B	NptII L, NptIIR	-
9.2	B	NptII L, NptIIR	-
10.1	A	1.2AS1, 35SPRO	-
10.2	A	1.2AS1, 35SPRO	-
10.3	A	1.2AS1, 35SPRO	-
11.1	B	NptII L, NptIIR	-
13.1	A	1.2AS1, 35SPRO	Y
13.2	A	1.2AS1, 35SPRO	-
13.4	A	1.2AS1, 35SPRO	-
14.1	A	1.2AS1, 35SPRO	-
14.2	A	1.2AS1, 35SPRO	-
14.3	A	1.2AS1, 35SPRO	-
14.4	A	1.2AS1, 35SPRO	-
14.5	A	1.2AS1, 35SPRO	-
14.6	A	1.2AS1, 35SPRO	-
14.9	A	1.2AS1, 35SPRO	-
14.10	A	1.2AS1, 35SPRO	-
14.11	A	1.2AS1, 35SPRO	-
14.12	A	1.2AS1, 35SPRO	-
14.13	A	1.2AS1, 35SPRO	-
14.14	A	1.2AS1, 35SPRO	-
14.15	A	1.2AS1, 35SPRO	-
14.17	A	1.2AS1, 35SPRO	-
14.18	A	1.2AS1, 35SPRO	-
14.19	A	1.2AS1, 35SPRO	-

Table 3.1 Summary of initial screening of *METII* transgenic lines listing line number; construct used for transformation (A = 1.2kb antisense; B = 570 bp sense); and PCR primers used in the screening procedure.

[a] Y = expression of *METII* was reduced as assayed by RT-PCR.

A) and 5 contained the 570 bp sense fragment (construct B). Each transformant was then analysed by PCR for either the 1.2 kb antisense transgene (construct A lines) or the *NptII* transgene (construct B lines) to ensure they contained the transgenes. A representative example of the PCR screening, showing three lines transformed with construct A, is shown in Figure 3.3. All 29 kanamycin-resistant lines containing construct A were positive for the 1.2 kb antisense transgene and all five lines containing construct B were positive for the *NptII* transgene (Table 3.1).

3.3.3 Analysis of *METII* expression level in transgenic lines

The level of *METII* expression in each of the 34 *METII* transgenic lines was initially analysed using reverse-transcriptase PCR (RT-PCR). As a control, the level of *METI* expression was also analysed and was compared to the level of *METII* expression in each line. Given that *METII* expression is unaffected in *METI* antisense lines (Genger, 2000) even though a 2.8 kb fragment of the *METI* cDNA, approximately 80 % identical to *METII* (Genger *et al.*, 1999) had been used to generate the *METI* antisense lines (Finnegan *et al.*, 1996), it was assumed that the *METII* constructs were unlikely to affect the level of *METI* expression, and that *METI* expression could therefore be used as a control. The *METII* primers for RT-PCR were designed to span introns 3 and 4, thus providing a size differential between products amplified from cDNA and genomic DNA (Figure 3.4). As the sequence of the RT-PCR primers is 100 % identical between the *METIIa* and *METIIb* genes, the RT-PCR analysis would detect transcripts from both genes.

An example of the RT-PCR screening of various lines is shown in Figure 3.5. The initial screening procedure revealed four lines with a potential decrease in *METII* expression; lines 6.3, 7.2, 13.1 and 6.2 (Figure 3.5, lanes 5, 7, 12 and 13). However, further replicates

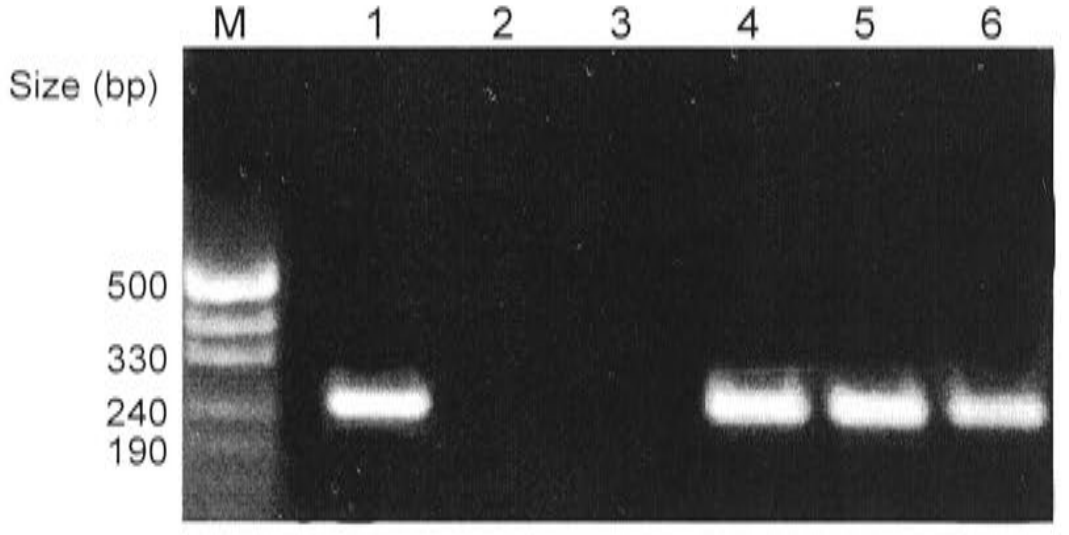


Figure 3.3 PCR screening of *METII* transgenic lines. The primers amplified a fragment encompassing part of both the 1.2 kb antisense fragment and the 35S promoter. Lane M, pUC19/*Hpa*II marker; lane 1, pJ35SN (1.2 kb) plasmid positive control; lane 2, water negative control; lane 3, C24 negative control; lanes 4-6, *METII* transgenic lines 6.1, 6.2 and 6.3.

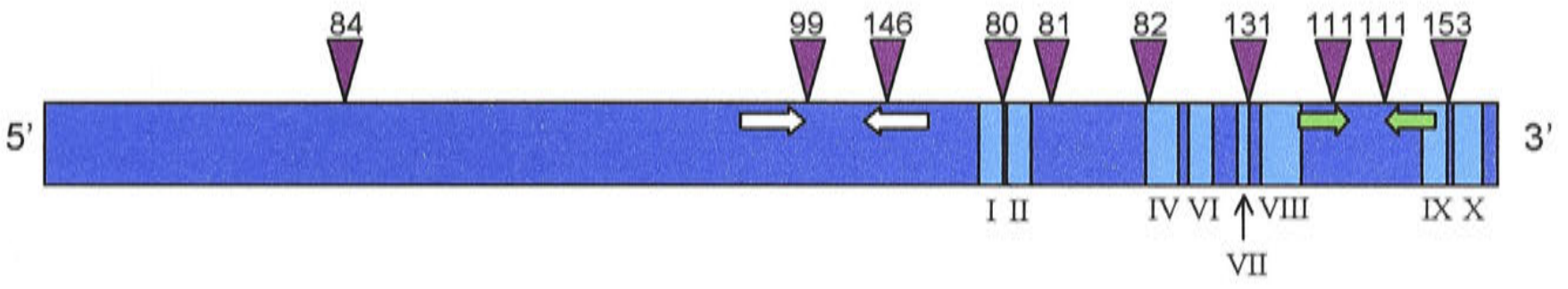


Figure 3.4 Location of *METII* conserved motifs, introns and primers used for RT-PCR and real time PCR. Introns 2 to 11 are indicated by ▼ and sizes of introns are given above in base pairs. Conserved methyltransferase motifs are indicated by pale blue boxes and Roman numerals. RT-PCR primer positions (RTXII and NEWI) are indicated by white arrows; real time PCR primer positions (METIIL and METIIR) are indicated by green arrows. Both sets of primers provide a size differential between products amplified from cDNA and genomic DNA. The RT-PCR cDNA product is 277 bp; the genomic product is 493 bp. The real time PCR cDNA product is 199 bp; the genomic product is 373 bp.

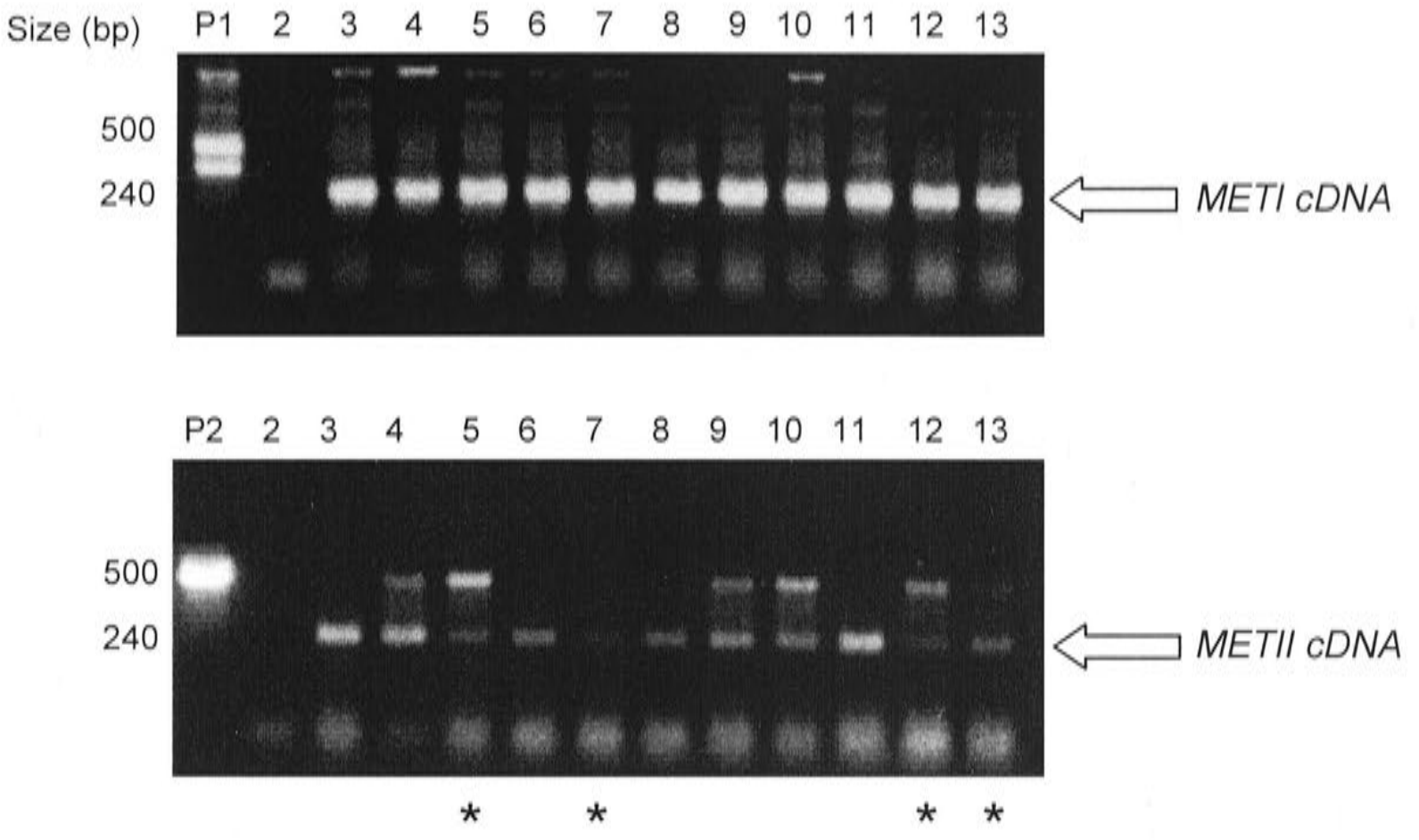


Figure 3.5 Initial RT-PCR screening of *METII* transgenic lines separated on 2% agarose gels. Upper panel, *METI* RT-PCR used as a control; lower panel, *METII* RT-PCR. Arrows indicate product from cDNA. The *METI* primers amplified several non-specific bands, presumably from contaminating genomic DNA, as they are also visible in the positive control. Although the *METI* 5' primer sequence is conserved among the four members of the *METI* class, the *METI* 3' primer sequence is not found in the other three genes, so the extra bands are unlikely to be amplified from *METIIa, METIIb* or *METIII* The major genomic DNA product for *METI* is 450 bp and the cDNA product is 205 bp. For *METII*, the genomic product is 493 bp and the cDNA product is 277 bp. Lane P1, C24 genomic DNA; lane P2, plasmid pJ35SN (1.2kb) positive control; lane 2, water negative control; lane 3, C24; lane 4, line 6.1; lane 5, line 6.3; lane 6, line 7.1; lane 7, line 7.2; lane 8, line 9.1; lane 9, line 10.1; lane 10, line 10.2; lane 11, line 11.1; lane 12, line 13.1; lane 13, line 6.2. Lanes marked * contain candidates for plants with reduced *METII* expression compared to C24.

(not shown) identified only lines 6.2, 7.2 and 13.1 as having a reproducible reduction in the level of *METII* expression. To estimate the extent of reduction in *METII* expression, PCR products from these three lines were transferred to a nylon membrane and hybridised to either the *METII* 1.2 kb fragment or the pBGSY8 *METI* probe (Figure 3.6). The ratio of *METI*:*METII* expression in C24 was then compared to the ratio of expression in the transgenic lines. The three lines had reduced levels of *METII* expression compared to wild type (Figure 3.7, blue bars). Line 7.2 had the lowest level of expression, with only 26.4 % of C24 *METII* expression levels. Lines 6.2 and 13.1 had 56.7 % and 52.9 % of C24 *METII* expression levels respectively. There is a very large error for line 6.2 (Figure 3.7, blue bar). Probable sources of error in the RT-PCR protocol could include pipetting very small volumes of concentrated RNA, variation in final reaction volumes due to evaporation from poorly sealed tubes, and spillage when loading gels.

To establish whether the apparent reduction in *METII* expression could be more reliably estimated, a real-time PCR cycler was utilised. Real-time PCR is both more sensitive and accurate than RT-PCR. Products do not need to be analysed by electrophoresis or Southern hybridisation, therefore reducing gel-associated errors. Due to the addition of the fluorescent SYBR-green dye which intercalates with DNA, the amplification of products can be visualised cycle by cycle; information about the amount of product produced is collected in the exponential amplification phase, thereby avoiding potential variation in the end product of identical reactions. To identify different products, for example from genomic DNA, cDNA or primer-dimer formation, melt curve analysis is performed. Products melt at different temperatures depending on several factors, including their length and GC content. In the experiments reported here, only those samples with a single peak in the melt curve analysis were used; any samples with contamination from genomic DNA or primer-dimer formation were excluded from further analysis.

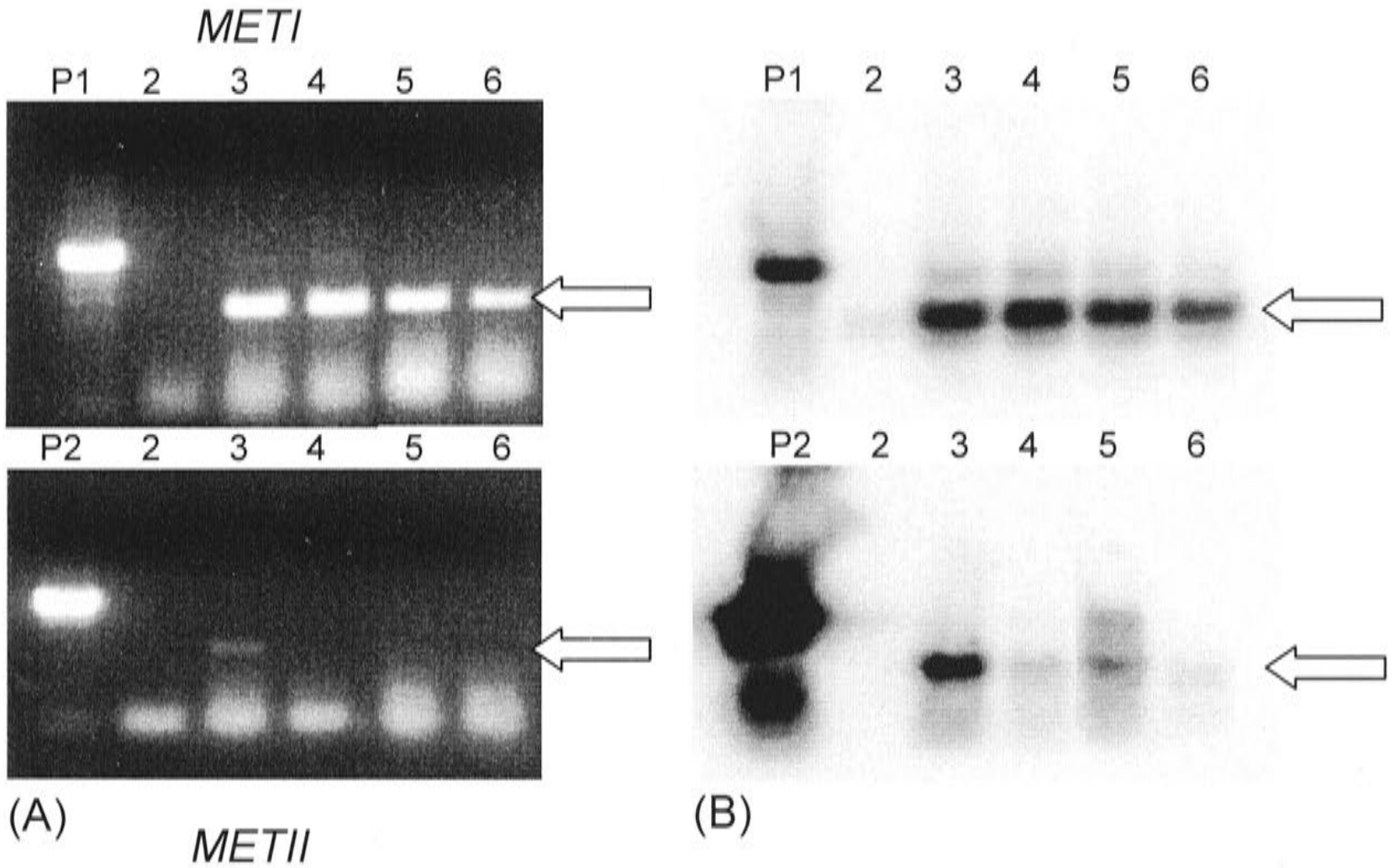


Figure 3.6 (A), RT-PCR of *METII* transgenic lines separated on 2% agarose gel. Upper panel, *METI* RT-PCR used as a control; lower panel, *METII* RT-PCR. Arrows indicate product from cDNA; other bands are products from genomic DNA contamination. Lane P1, C24 genomic DNA; lane P2, plasmid pJ35SN (1.2kb) positive control; lane 2, water negative control; lane 3, C24; lane 4, line 7.2; lane 5, line 13.1; lane 6, line 6.2.
(B), Southern analysis of *METI* and *METII* RT-PCR gels shown in (A). Arrows indicate product amplified from cDNA. The *METI* probe was pBGSY8 (see section 3.2.2). The *METII* probe was the PCR product amplified from the pJ35SN plasmid containing the 1.2 kb *Hin*dIII fragment, using the same primers as those used in the RT-PCR reactions.

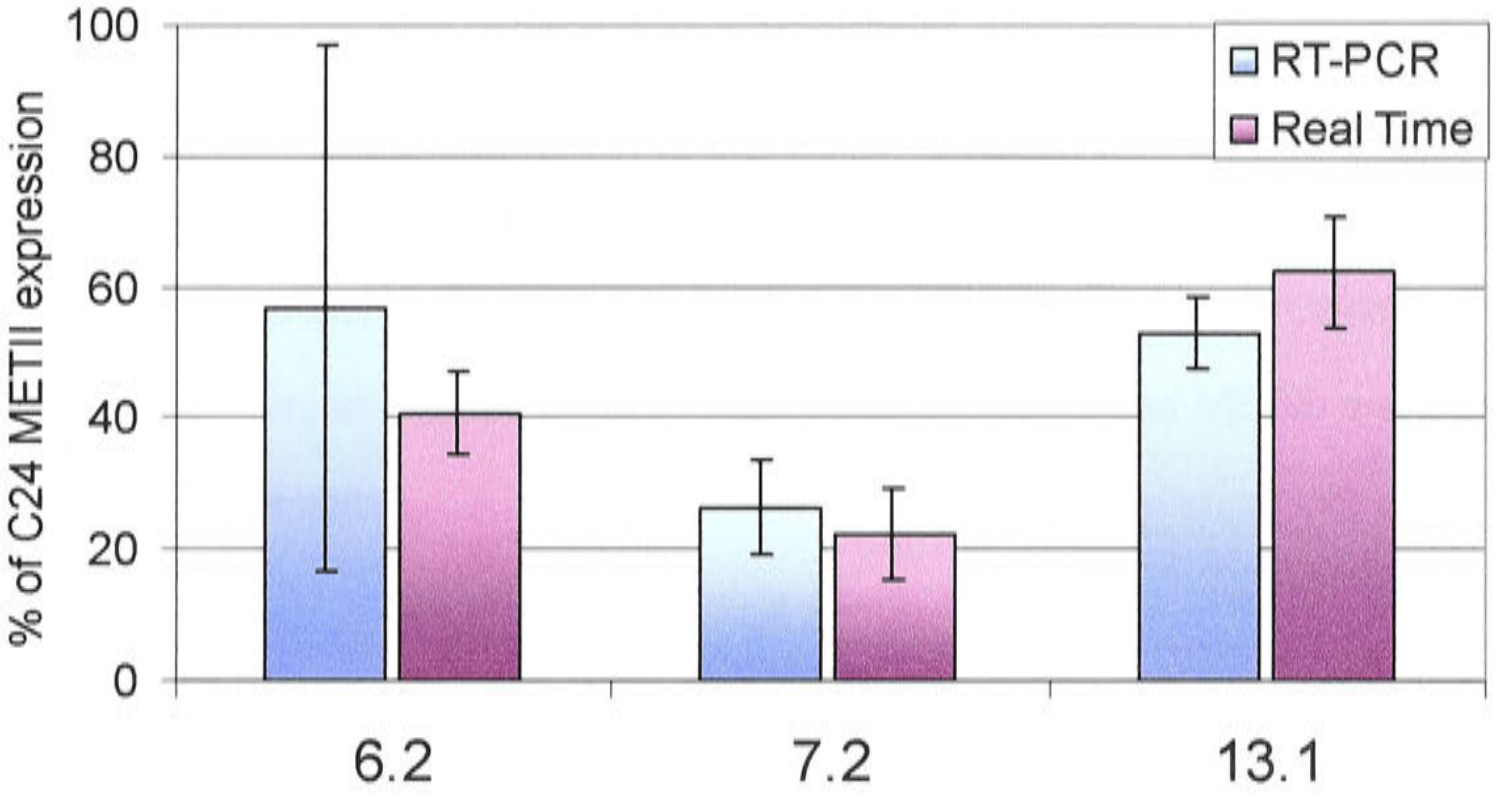


Figure 3.7 Level of *METII* expression in three *METII* transgenic lines, expressed as a percentage of C24 *METII* expression ± standard error. The level of C24 *METII* expression is defined as 100%. Blue bars, RT-PCR results. Purple bars, real time PCR results. Results presented are the mean of three replicates.

The primers used to amplify *METI* were the same as those used for *METI* RT-PCR (section 3.2.2.1). The *METII* primers spanned intron 9 and 10 (Figure 3.4). The primers were designed in an attempt to amplify only *METIIa* but had only two mismatches with the *METIIb* sequence. Melt curve analysis revealed only one cDNA product in the *METII* reactions. As *METIIb* is 94% identical to *METIIa* in the region amplified by the real time PCR primers and only 19 bp different in length, the *METIIa* and *METIIb* products would be very difficult to identify separately using melt curve analysis (I Wilson, personal communication) and it must be assumed that both *METIIa* and *METIIb* are amplified in the real time PCR assay.

It must also be assumed that the *METII* constructs used for transformation of *Arabidopsis* affected the expression of both *METIIa* and *METIIb*. Only 21 bp of homologous sequence is sufficient for post-transcriptional gene silencing to occur (Hamilton and Baulcombe, 1999; Waterhouse et al, 2001); there are 15 regions of 21 or more bp of homologous sequence in the *METII* 1.2 kb fragment and 12 such regions in the 570 bp *METII* fragment used to make the constructs. The high degree of similarity between the two genes suggests that they may be functionally redundant, although they may have different expression patterns. Thus there would be benefit in silencing both *METIIa* and *METIIb,* to increase the chance of uncovering a function for them. As they cannot be distinguished at this point, *METIIa* and *METIIb* will be jointly referred to as *METII* throughout the remainder of this chapter.

Using the real-time PCR system, the level of *METII* expression was again compared to that of *METI* and the ratio of *METI:METII* expression in C24 was compared to the ratio of *METI:METII* expression in the transgenic lines. Expression of *METII* was reduced in all

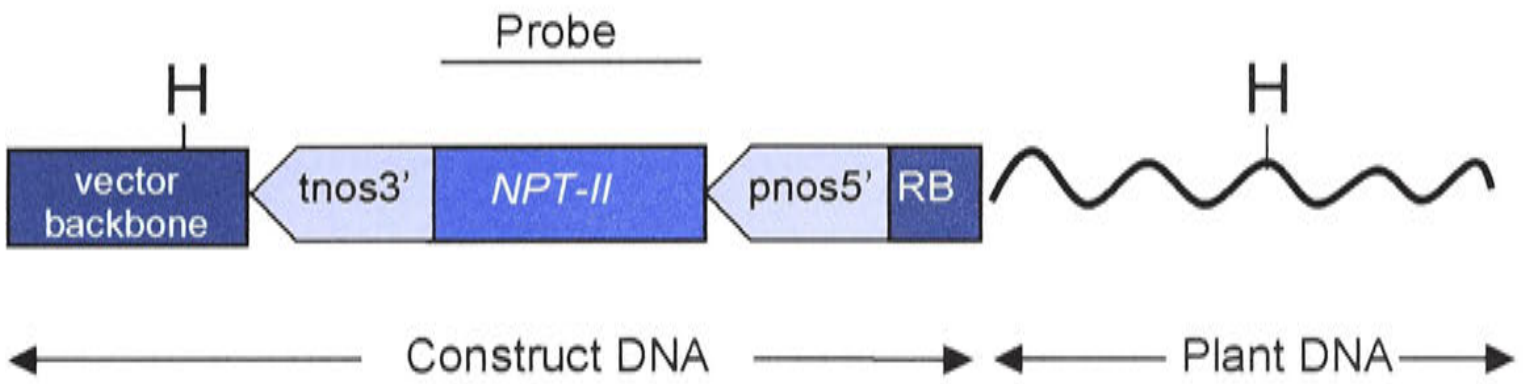
three transgenic lines compared to C24, over a similar range to that seen in the RT-PCR experiments. Line 7.2 again had the lowest level of expression, with only 22.3 % of C24 *METII* expression levels. Line 6.2 had 40.8 % of wild type levels and line 13.1 had the least reduction, with 62.3 % of wild type levels (Figure 3.7). The reproducibility of the values recorded for *METII* expression levels using real-time PCR was greatly increased compared to the RT-PCR results, especially for line 6.2. The real-time PCR protocol was designed to avoid pipetting-associated errors, by ensuring that the minimum volume pipetted was five microlitres.

Each of the remaining 31 *METII* transgenic lines which had been previously screened by RT-PCR were re-screened using the real-time PCR system. No further lines with a greater than 40 % reduction in *METII* expression compared to C24 were identified (results not shown). Hence only the three lines described above, which have a range of 40-80 % decrease in *METII* expression, were characterised in more detail.

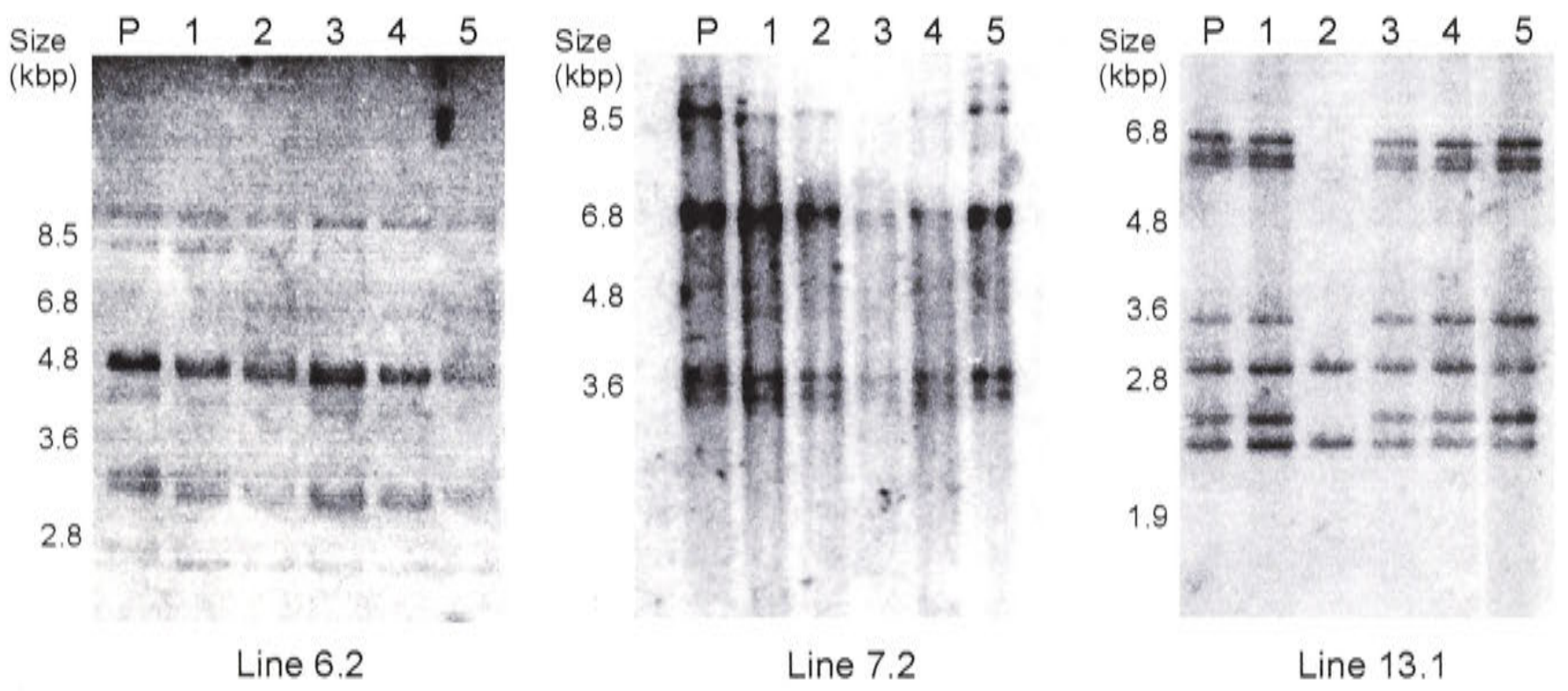
3.3.4 Transgene copy number and progeny testing of *METII* transgenic lines

The *METII* transgenic lines were analysed to determine the number of T-DNA copies which had inserted into each line. DNA was extracted from five individual T2 plants and a pooled sample of the same plants and digested with *Hind*III and hybridised to an *NptII* probe (Figure 3.8a). Each line had multiple copies of the transgene (Figure 3.8b). Line 6.2 had 6-7 copies and line 7.2 had 7-8 copies of the *NptII*/left border fragment. As all plants tested contained every copy, it is probable that these transgenes had inserted at a single locus. However, it is also possible that the insertion events could have been at two loci, as DNA from 20 plants would need to be analysed to be 99 % sure that there were no plants which were null at one of two loci. These two lines were subsequently subjected to

Figure 3.8 (A), DNA from *METII* transgenic plants with reduced levels of *METII* expression was digested with *Hin*dIII and hybridized to the *Npt*-II probe. One *Hin*dIII site (H) is located within the construct. The next *Hin*dIII site is located within the plant DNA. Multiple bands on Southern blots indicate multiple copies of the transgene. **(B),** DNA isolated from five individual plants and a pooled sample (P) of the same five plants was digested with *Hin*dIII, transferred to a nylon membrane and hybridized to the *Npt-II* probe. The smallest hybridizing fragment that would be expected, if the first *Hin*dIII site was immediately adjacent to the RB fragment, would be approximately 2.6 kb.


Probe
H
H
vector backbone
tnos3'
NPT-II
pnos5'
RB
Construct DNA
Plant DNA

(A)


Size
P 1 2 3 4 5
(kbp)
8.5
6.8
4.8
3.6
2.8
Line 6.2
Size
P 1 2 3 4 5
(kbp)
8.5
6.8
4.8
3.6
Line 7.2
Size
P 1 2 3 4 5
(kbp)
6.8
4.8
3.6
2.8
1.9
Line 13.1

(B)

progeny testing by growing T2 seedlings on media containing the antibiotic kanamycin (Figure 3.9). After three weeks growth, the numbers of kanamycin-resistant and kanamycin-sensitive seedlings were counted. If insertion of the transgenes had been at a single locus, approximately 25 % of the seedlings would be sensitive to kanamycin; if insertion was at two loci, approximately 6 % of seedlings would be sensitive. In line 6.2, 14 % of seedlings were sensitive; in line 7.2, 17 % of seedlings were sensitive (Table 3.2). A χ^2 test showed that with the number of plants screened, these ratios are statistically significantly different to a ratio of 3:1 (Table 3.2). However, if insertion was at two loci, the ratios expected would have been 15:1, whereas they are much closer to a 3:1 ratio. In conjunction with the Southern analysis of copy number, the ratios are suggestive of insertion at a single locus, but a larger number of plants would need to be screened to be sure of this.

The third line analysed, line 13.1, had 7 copies of the transgene (Figure 3.8). The Southern analysis suggested that these had inserted in at least two loci, with five copies at one locus, and two at a separate locus. When progeny testing of T2 seedlings of line 13.1 was carried out, it appeared that all 114 progeny tested were resistant to kanamycin (Figure 3.9; Table 3.2). If, as the Southern analysis suggested, the transgene had integrated at two loci, 6 % of the seedlings would have been expected to be sensitive, or seven of the 114 seedlings tested. If integration had occurred at three loci, only one or two of the 114 seedlings would be sensitive. The number of plants tested is too small to be sure whether the transgene had inserted in two or three loci. A χ^2 test could not be carried out on this data as there were zero seedlings in one of the categories.

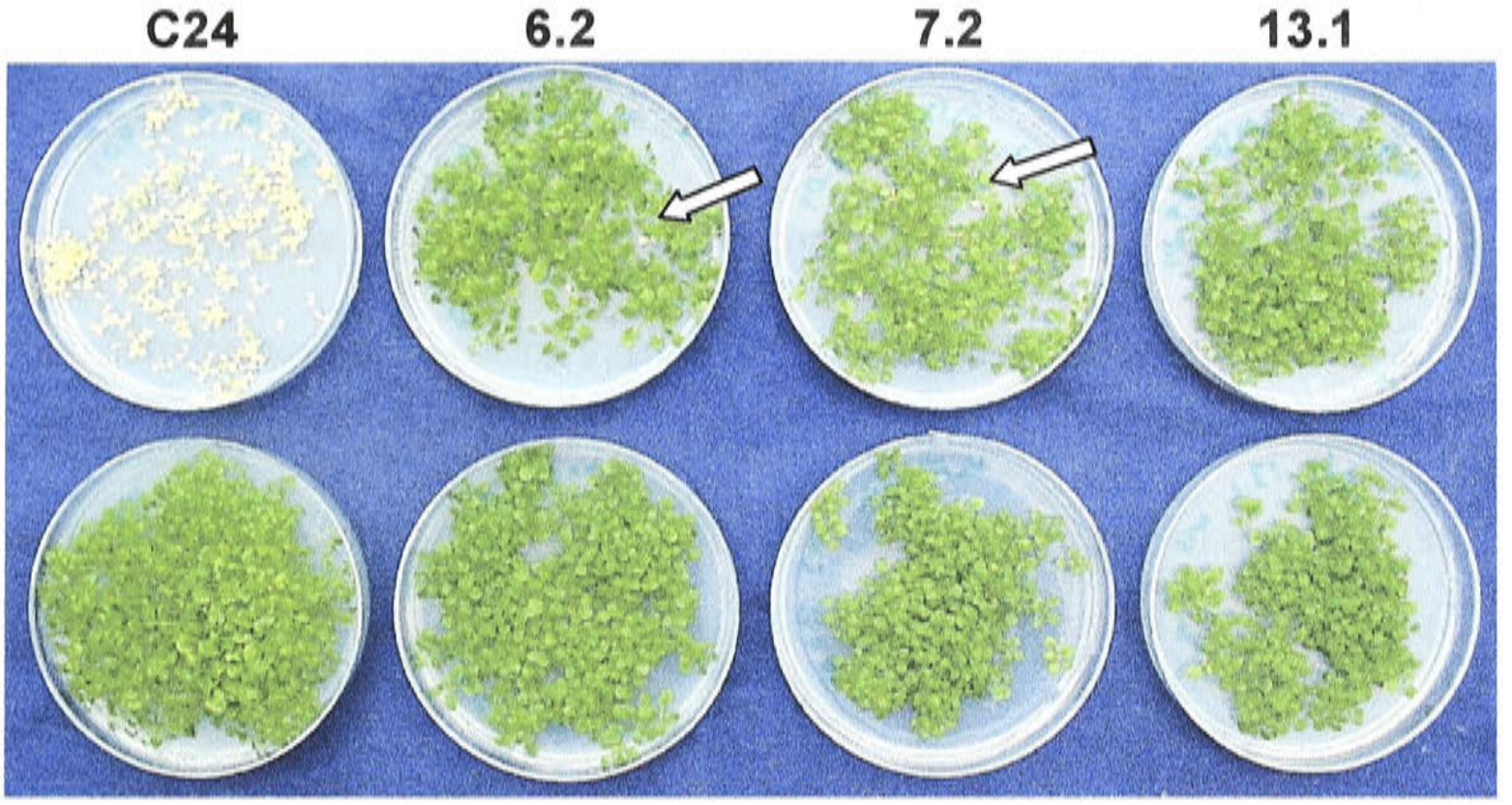


Figure 3.9 Three week old seedlings of C24 and three *METII* transgenic lines with reduced levels of *METII* expression grown on MS media with kanamycin (top row) or without kanamycin (bottom row). Kanamycin sensitive plants are bleached, and development has stopped at the two leaf stage. Arrows indicate two representative kanamycin sensitive seedlings; most of them are obscured from view by the larger resistant seedlings.

Line	Number of resistant: sensitive seedlings	Calculated ratio	χ^2 value	p value
6.2	127:20	6.3:1	9.58	< 0.01
7.2	113:23	4.9:1	4.32	< 0.05
13.1	114:0	-	-	-

Table 3.2 Results of progeny testing of T2 plants of three *METII* transgenic lines. Plants were grown on MS media containing 50 μg/mL kanamycin for 3 weeks.

χ^2 value for $p < 0.05 = 3.84$; χ^2 value for $p < 0.01 = 6.64$.

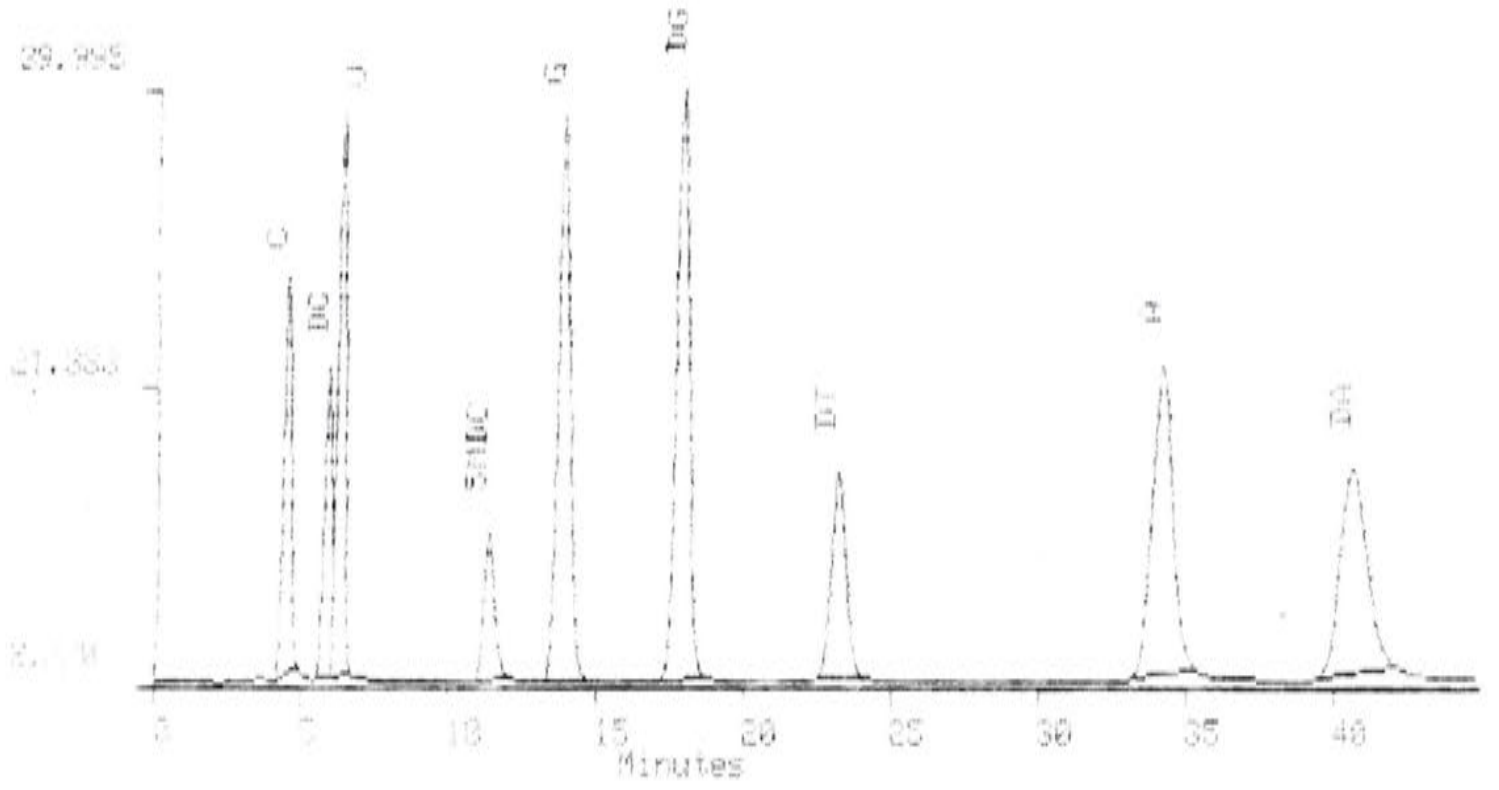
3.3.5 Analysis of DNA methylation levels in *METII* transgenic lines

3.3.5.1 Analysis of genomic DNA methylation levels

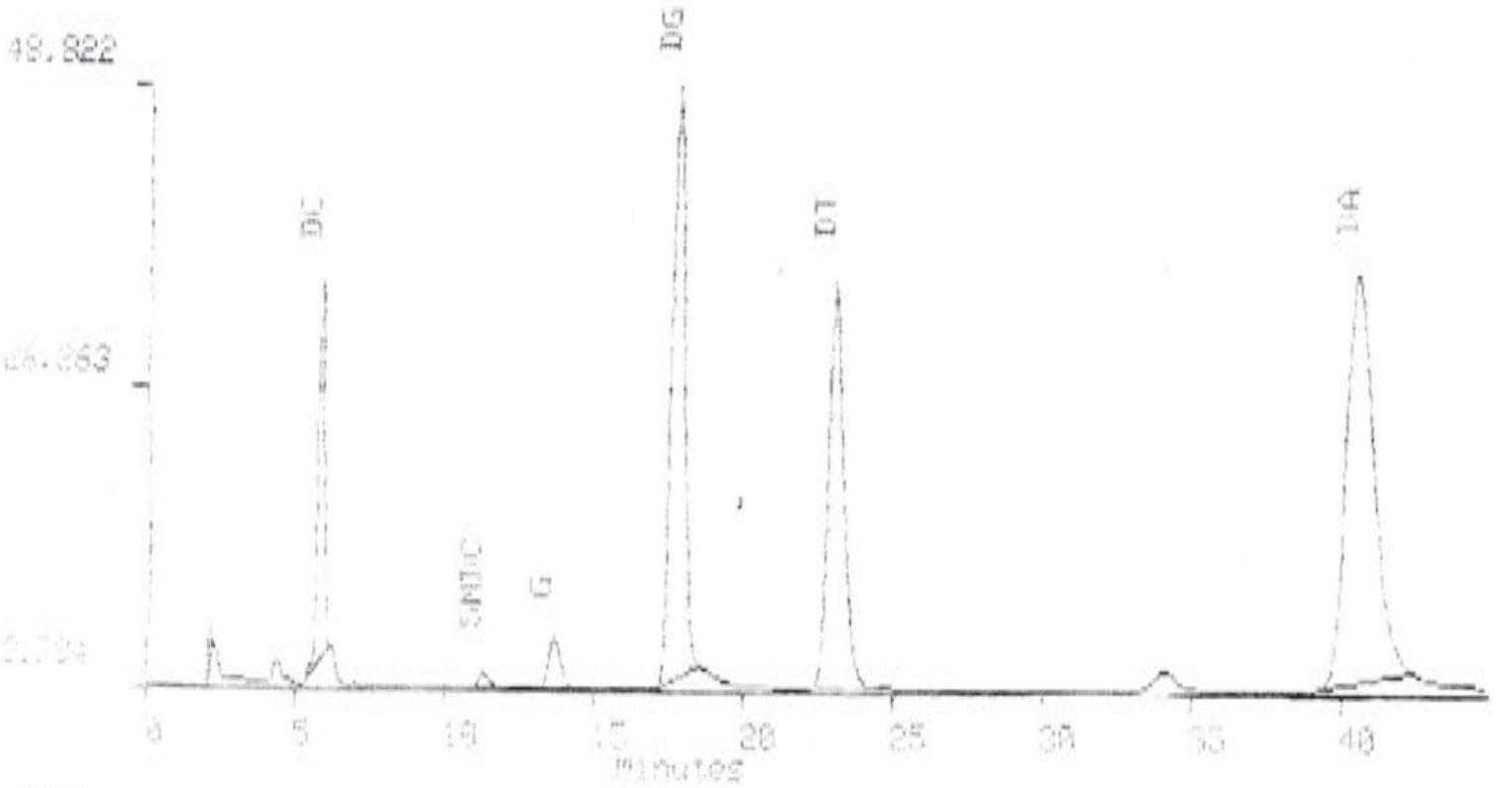
To determine whether reducing the level of *METII* expression by up to 80 % had any effect on methylation levels, the global levels of methylation in each *METII* transgenic line were analysed using RP-HPLC. RP-HPLC analysis provides a measurement of the proportion of each major and modified base in a sample of DNA. As RP-HPLC had not been previously performed in this laboratory, the protocol was developed based on the method of Gehrke *et al.* (1984). DNA and RNA standards, including methyl-deoxycytosine (^{m}C) were first separated individually to determine the retention time of each nucleoside. A mixture of all the standards was then applied to the column to ensure that each nucleoside eluted separately as a clearly resolved peak (Figure 3.10).

The HPLC system was initially utilised to determine the levels of ^{m}C in wild type C24 genomic DNA. A representative trace of a C24 sample separated on the RP-HPLC column is shown in Figure 3.10b. The average level of ^{m}C in triplicate samples of C24 DNA was (7.11 $\pm$ 0.48) % (Figure 3.11); the range of values was from 6.63 % to 7.59 %, which equates a 7 % deviation from the average. This overlaps with published values of 6.4-6.6 % for the Columbia ecotype (Kakutani *et al.*, 1996; Ronemus *et al.*, 1996). When other ecotypes of *Arabidopsis* were tested using this system, a range of values was observed (Figure 3.11), indicating that there is a difference in genomic methylation levels between different wild types. This is consistent with the observation that the extent of ^{m}C in ribosomal repeat DNA varies among ecotypes (Riddle and Richards, 2002).

The methylation levels of a *METI* antisense line (AMT, line T3 10.5) had been previously estimated using a thin-layer chromatography (TLC) assay to have 13.3 % of wild type



(A)



(B)

Figure 3.10 Sample RP-HPLC traces. X-axis = retention time (minutes). Y-axis = absorbance at 254nm. **(A)**, mixture of DNA and RNA standards. **(B)**, C24 control sample. A small amount of guanosine from contaminating RNA is visible in this sample.

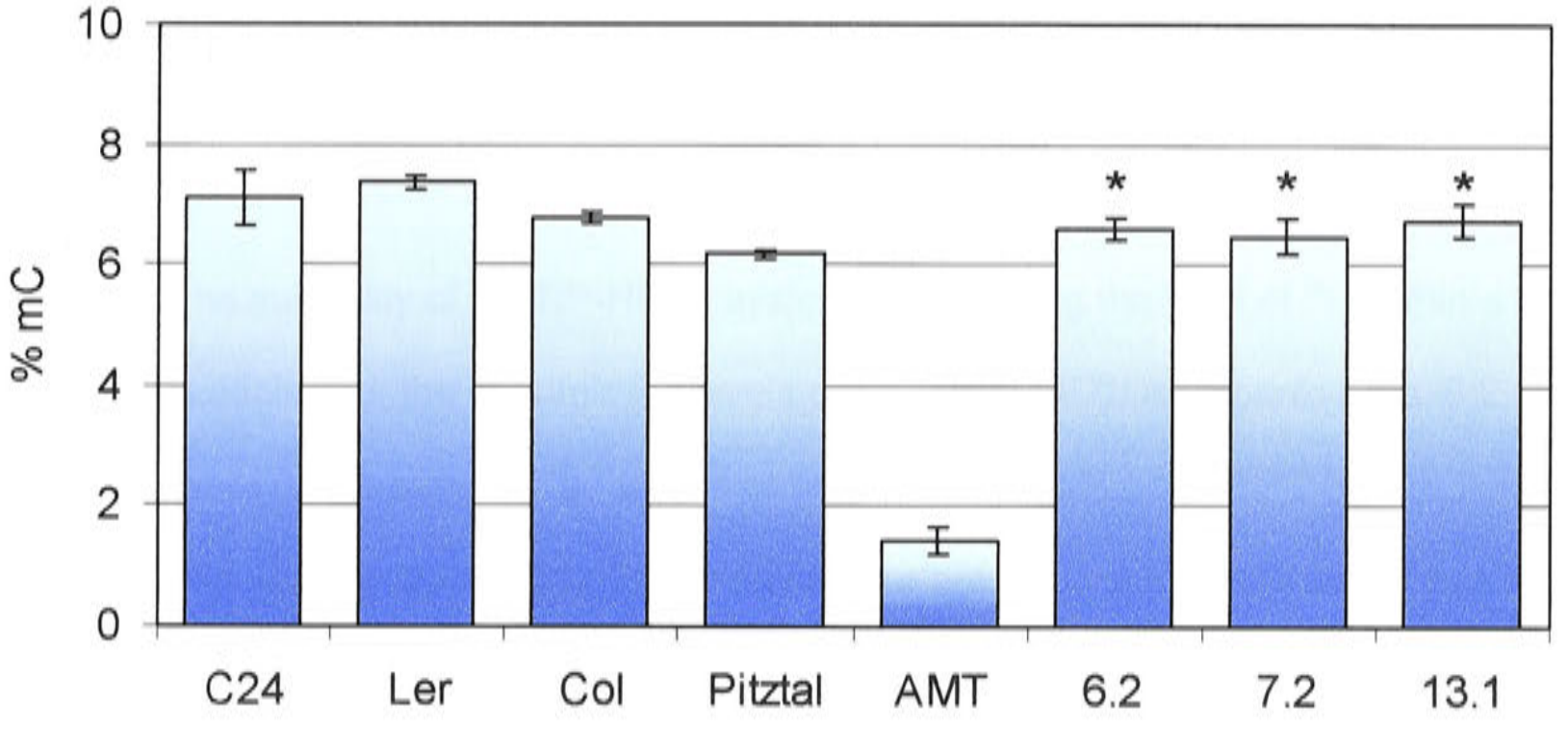


Figure 3.11 Genomic levels of methyl cytosine (mC) in four ecotypes of *Arabidopsis, METI* antisense plants and *METII* transgenic plants as determined by RP-HPLC analysis. Each bar represents the mean of at least three replicates. Ler, Landsberg *erecta;* Col, Columbia; AMT, *METI* antisense line T3 10.5 (Finnegan *et al.,* 1996); 6.2, 7.2 and 13.1, three *METII* transgenic lines with reduced levels of *METII* expression.

* amount of genomic mC not significantly different from wild type C24; $p>0.05$

methylation levels in the subset of CG dinucleotides that occur within *Taq*I sites (Finnegan *et al.*, 1996). The same AMT line was analysed using the RP-HPLC system to ensure that a decrease in methylation could be reliably detected, and was estimated to have 19 % of the genomic mC level of C24 (Figure 3.11). This level is higher than the published value of 13.3 %; however, the AMT line is decreased in methylation mostly at CG sites (Kishimoto *et al.*, 2001) and the RP-HPLC analysis will have detected methylation at every other site, accounting for this difference.

Once the suitability of the RP-HPLC system for detecting the level of ^{m}C within ± 7 % had been established, the genomic ^{m}C levels of the three *METII* transgenic lines, 6.2, 7.2 and 13.1, were then assayed. The values obtained varied from 6.49 % ^{m}C for line 7.2 to 6.73 % ^{m}C for line 13.1 (Figure 3.11). These values are not significantly different to that of the wild-type C24 as determined by using Student's T-test ($p>0.05$). Therefore, reducing the level of *METII* expression by up to 80 % had no significant impact on genomic methylation levels.

3.3.5.2 Analysis of DNA methylation levels within specific sequence contexts

The levels of DNA methylation of the three *METII* transgenic lines within certain sequence contexts was measured using methylation-sensitive enzyme assays. The restriction enzyme pair *Msp*I and *Hpa*II are commonly used to assay methylation of CG dinucleotides. Both enzymes recognise the sequence CCGG; *Msp*I cuts C^{m}CGG but not ^{m}CCGG, whereas its isoschizomer *Hpa*II will only cut when both cytosines are unmethylated or when the 5' C is hemimethylated (Kessler *et al.*, 1985; Nelson & McClelland, 1991). Therefore, a DNA sample digested with *Hpa*II will remain mostly uncut if the majority of CCGG sites are methylated. C24 DNA was digested separately with *Msp*I

and with *Hpa*II and was then hybridised to a 180 bp centromeric repeat probe (Martinez-Zapater *et al.*, 1986). The *Msp*I-digested sample shows a high degree of digestion of centromeric repeat DNA (Figure 3.12, lane 1). Hybridisation in the *Hpa*II-digested sample is concentrated in the poorly digested higher molecular weight fraction, indicating that many CCGG sequences are methylated at the internal cytosine in C24 DNA (Figure 3.12, lane 2).

A *METI* antisense line with a 30 % decrease in CG methylation relative to C24, as measured by TLC assays (EJ Finnegan, personal communication), was next assayed using *Hpa*II digestion to ensure that this level of reduction in DNA methylation could be detected using this method. The *METI* antisense DNA sample is digested by *Hpa*II (Figure 3.12, lane 6), indicating that this technique is suitable for detecting at least a 30 % decrease in CG methylation. DNA from the *METII* transgenic lines 6.2, 7.2 and 13.1 was then digested with *Hpa*II. Nearly all the hybridisation in these samples was concentrated in the poorly digested fraction (Figure 3.12, lanes 3-5). This result indicates that these lines have no detectable decrease in CG methylation of centromeric repeat DNA compared to C24.

Methylation at CNG sites (where N = T) can be assayed using the enzyme *Pvu*II. This enzyme recognizes the sequence CAGCTG, but cuts only when the internal cytosine is unmethylated. DNA samples digested with *Pvu*II were hybridised to a 1.7 kb 28S ribosomal repeat probe (Campbell *et al.*, 1992). C24 DNA digested with *Pvu*II hybridised to the ribosomal probe only in the poorly digested fraction (Figure 3.13, lane 1) indicating that many CTG sites in C24 ribosomal DNA are methylated. DNA from the *METII* lines 6.2, 7.2 and 13.1 likewise remained poorly digested by *Pvu*II, suggesting they had no detectable

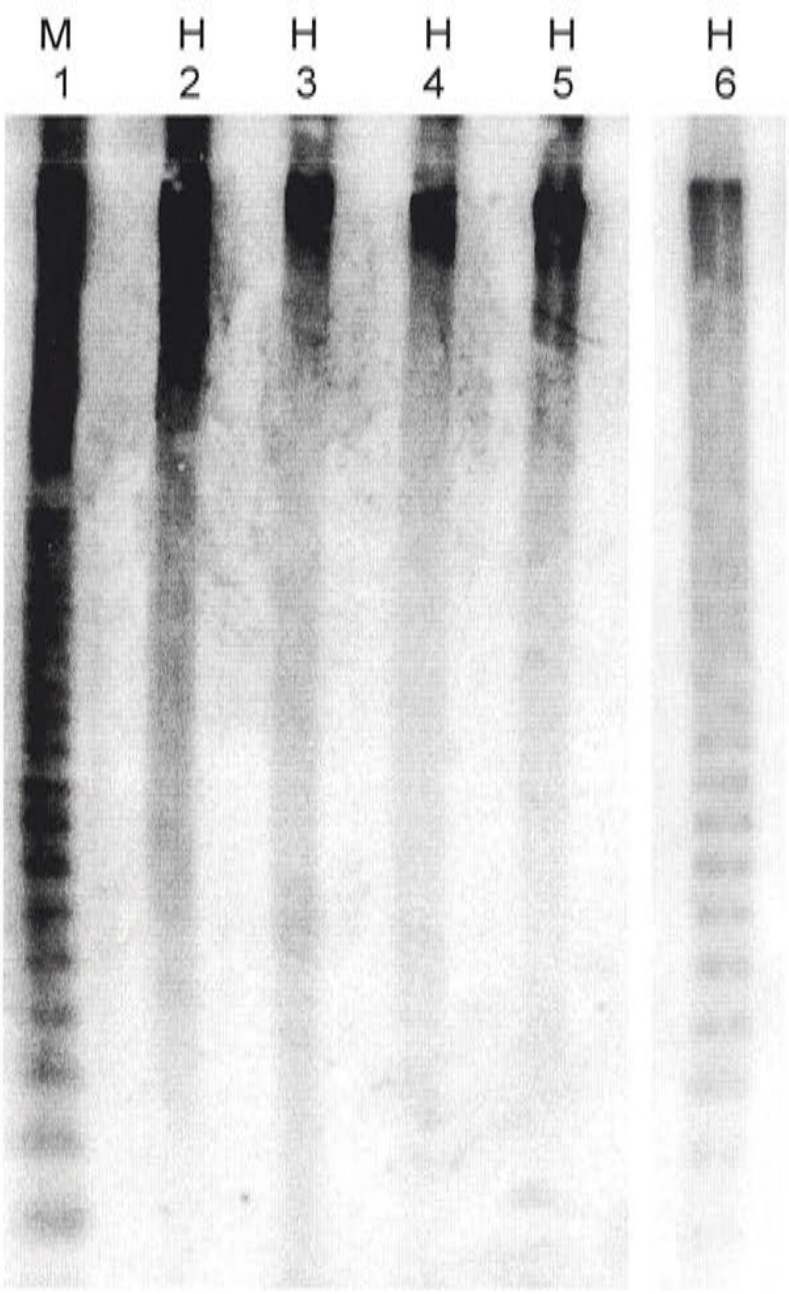


Figure 3.12 Analysis of methylation at CG sites in DNA from C24, three *METII* transgenic lines with reduced levels of *METII* expression and a *METI* antisense line. DNA was digested with *Msp*I (M) or *Hpa*II (H) and hybridized to a 180 bp centromeric ribosomal repeat probe. Lanes 1 and 2, C24; lane 3, line 6.2; lane 4, line 7.2; lane 5, line 13.1; lane 6, *METI* antisense line with 30% decrease in CG methylation compared to wild type.

Figure 3.13 Analysis of methylation at CTG sites in DNA from C24 and three *METII* transgenic lines with reduced levels of *METII* expression. DNA was digested with *Pvu*II and hybridized to a 28S ribosomal repeat probe. Lane 1, C24; lane 2, line 6.2; lane 3, line 7.2; lane 4, line 13.1.

decrease in CTG methylation of 28S ribosomal repeat DNA compared to C24 (Figure 3.13, lanes 2-4).

3.3.6 METII may have different targets for methylation than METI

The results described above show that when *METII* expression is reduced by up to 80 %, no change in either overall methylation levels or at CG or CTG sites in repeated sequences can be detected. This does not necessarily mean that METII is a non-functional enzyme. It is possible that highly specific sites are the target/s of METII, but that these sites may not occur frequently enough to be detected by the assays that were used, or may not occur in the regions of DNA assayed by the probes that were chosen.

General observations of the phenotype and gross morphology of the *METII* lines did not immediately suggest any roles for METII in wild type plants. The plants were of normal stature; leaf shape and size, floral development and fertility did not seem to be affected. In contrast, *METI* antisense plants are affected in all these criteria. One of the characteristics of *METI* antisense plants that was not immediately obvious, however, was their ability to rescue the phenotype of mutations in *fis* (***f**ertilisation **i**ndependent **s**eed*) genes. The products of the *FIS1* and *FIS2* genes repress seed development until after fertilisation has occurred; mutations in these genes result in arrested embryo development, causing the seeds to atrophy or shrivel (Chaudhury *et al.*, 1997). FIS1 is similar to the Polycomb group proteins E(z) in *Drosophila* and CLF in *Arabidopsis* (Goodrich *et al.*, 1997), which are regulators of homeotic gene expression. FIS2 is a WD40 repeat protein (Luo *et al.*, 1999, 2000) that is homologous to the PcG proteins Su(Z)12 and VRN2 (Birve *et al.*, 2001; Gendall *et al.*, 2001). When *fis1* and *fis2* mutants are fertilised with pollen from a *METI* antisense plant, the phenotype is rescued and the seeds develop normally (Luo *et al.*,

2000), thought to be due to demethylation-induced activation of an unidentified gene or genes downstream of the *FIS* genes.

As mentioned, it is possible that METII methylates highly specific sites. The high degree of sequence similarity between *METI* and *METII* suggests that the two enzymes could be potentially redundant, and that some of the targets of METII regulation might be the same as those of METI. To investigate this possibility, the two lines with the greatest reduction in *METII* expression, lines 6.2 and 7.2, were crossed to the *fis1* and *fis2* mutants. C24 and *METI* antisense plants were crossed to the *fis* mutants as negative and positive controls respectively. The *fis* mutants were *FIS/fis* heterozygotes, thus after a cross to a wild type *FIS/FIS* plant, half of the F1 seeds should display an inviable, shrivelled phenotype.

When C24 pollen was used to fertilise the *fis1* mutants, half of the seeds were shrivelled (Figure 3.14a). When *METI* antisense plants were used as pollen donors, all of the seeds developed normally, as previously reported (Figure 3.14b; Luo *et al.*, 2000). When the *METII* transgenic lines were used to fertilise the *fis1* mutant, approximately half the F1 seed was inviable (Figure 3.14c, d). The same result was observed in crosses made to *fis2* mutants (results not shown). As pollen from the *METII* lines did not rescue the *fis1* or *fis2* phenotype, it appears that METII is not involved in the regulation of the genes downstream of *FIS1* or *FIS2* that METI regulates, and so at least in this instance, does not have the same targets for methylation as METI. This is consistent with the lack of demethylation at CG sites, the preferred target of METI (Finnegan *et al.,* 1996; Kishimoto et al., 2001), in the *METII* transgenic lines, and suggests that *METII* is not redundant with *METI*.

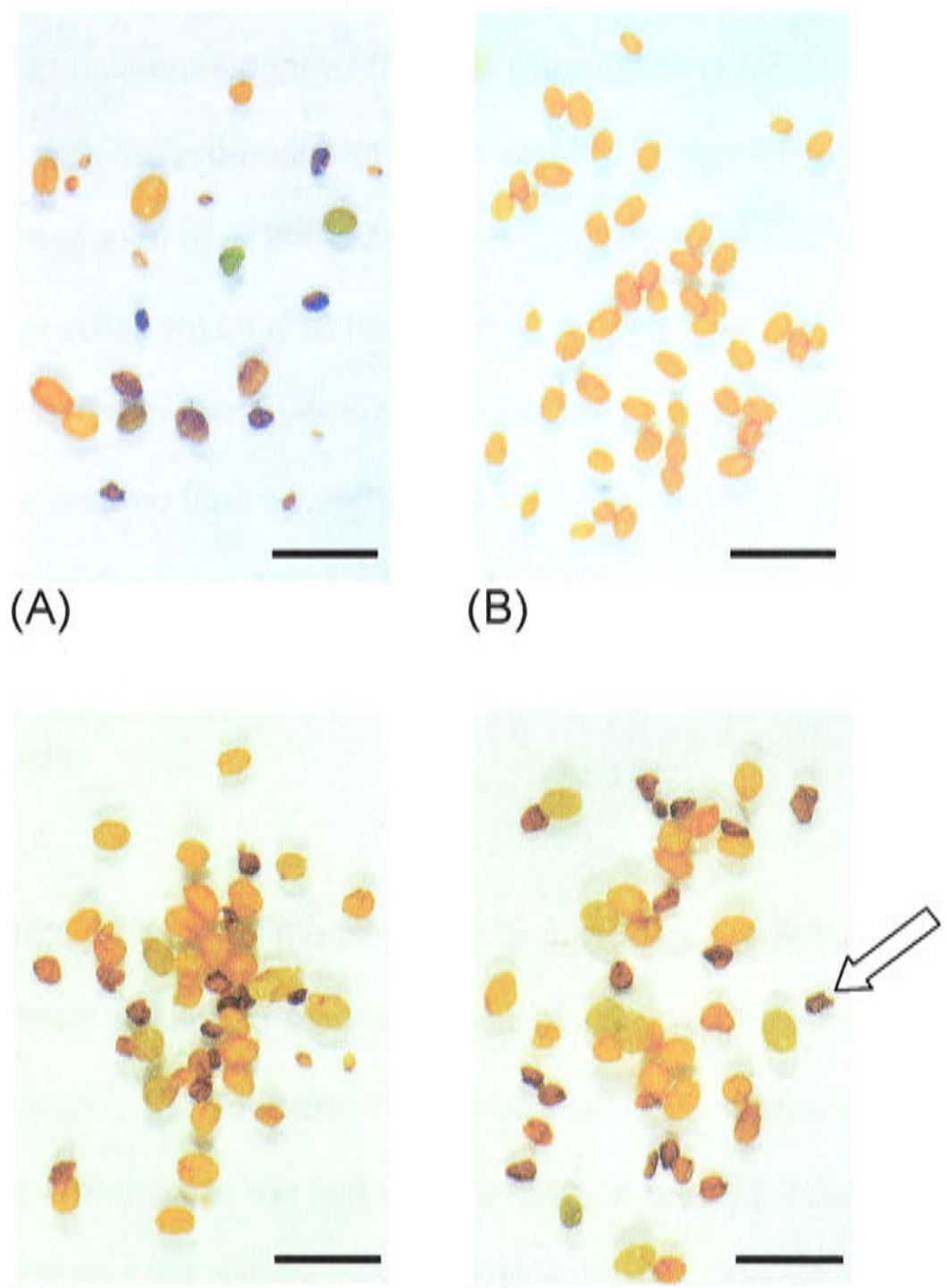


Figure 3.14 F1 seed resulting from crosses between C24, a *METI* antisense line or two *METII* transgenic lines and heterozygous *fis1* mutants, examined under a dissection microscope. All seeds in the *fis1 x METI* line are viable, whereas approximately 50% of seed in the other crosses are inviable. Arrow indicates a representative inviable shriveled seed. **(A)**, C24 x *fis1*. **(B)**, *METI* antisense line T3 10.5 x *fis1*. **(C)**, *METII* line 6.2 x *fis1*. **(D)**, *METII* line 7.2 x *fis1*. Bar = 3 mm.

3.3.7 Reducing *METII* expression alters flowering time

Plants carrying an antisense against the methyltransferase *METI* (AMT) flower earlier than wild type plants, with the promotion of flowering being proportional to the decrease in methylation (Finnegan *et al.*, 1998). Wild type C24 plants flower early in response to vernalisation, a process which also results in a decrease in methylation (Finnegan *et al.*, 1998). AMT plants retain some vernalisation response, even when unvernalised AMT plants flower at the same time as vernalised C24 (Finnegan *et al.*, 1998). Therefore, it is possible that other sites important for the vernalisation response exist, and that their methylation status is controlled by an enzyme/s other than METI; perhaps METII might be involved in this role.

To analyse the role of *METII* in the transition to flowering, the flowering time of the three segregating T2 lines which had reduced levels of *METII* expression, lines 6.2, 7.2 and 13.1, was compared to the flowering time of C24 wild type plants in long day conditions in two separate experiments. In the first experiment plants were grown in individual test tubes without kanamycin and the results include data from homozygous, heterozygous and null plants. In the second experiment, plants were grown on large Petri plates and the results include data from kanamycin-resistant (homozygous and heterozygous) plants only, not nulls. As the antisense and sense constructs have a dominant effect, the phenotype of heterozygotes should be roughly equivalent to that of homozygotes. *METI* antisense-null plants inherited low methylation levels and flowered as early as homozygous and heterozygous siblings, indicating that low methylation caused the promotion of flowering, and not the presence of the transgene (Finnegan *et al.*, 1998). Therefore, if demethylation caused by the *METII* transgene affects flowering time, plants which are null for the *METII* constructs should also be affected.

3.3.7.1 Flowering is promoted in two METII transgenic lines

When plants were grown in individual test tubes, C24 flowered after producing 33 leaves (Table 3.3, Experiment 1). The *METII* lines were also tested under the same conditions. Line 6.2 flowered with 29.9 leaves, and line 7.2 flowered after producing 27.9 leaves. The promotion of flowering in these two lines compared to C24 is statistically significant. The third line, 13.1, flowered after producing 31.8 leaves, which is not significantly different to the number of leaves produced by C24. Therefore, in two of the *METII* lines, flowering is promoted relative to C24. This result correlates well with the levels of reduction in *METII* expression; line 7.2 has the greatest reduction in *METII* expression (Figure 3.7) and has the greatest promotion of flowering. In contrast, line 13.1, which has the smallest decrease in *METII* expression (Figure 3.7), does not flower significantly earlier than C24.

In the second experiment, when the plants were grown on large Petri plates, C24 flowered after producing 21.1 leaves (Table 3.3, Experiment 2). This is much earlier than in experiment 1, where C24 flowered with 33 leaves (Table 3.3, Experiment 1). The reason for this difference has not been determined but is likely to reflect different growth conditions. Variables between the two experiments included seed age, different growth cabinets, and the amount of growth media used. The light intensities were comparable between the two experiments but different brands of fluorescent tubes were used. The spectral properties of these tubes may have differed, which can affect flowering time (Finnegan *et al.*, 1998; Genger, 2000). In addition, although the growth room temperature was 21 °C in both experiments, the plates were situated closer to the lights than the test tubes were and hence may have been slightly warmer.

Line/Treatment	Experiment 1 TLN ± SE (n)[a]	Experiment 2 TLN ± SE (n)[a]
C24	33.00 ± 0.75 (19)	21.11 ± 0.70 (26)
6.2	29.90 ± 0.99 (21)[b]	18.50 ± 0.66 (28)[b]
7.2	27.85 ± 1.13 (20)[c]	15.53 ± 0.99 (15)[c]
13.1	31.77 ± 1.45 (13)	21.21 ± 0.70 (24)
C24 + vernalisation	12.68 ± 0.50 (19)	10.43 ± 0.20 (21)
6.2 + vernalisation	13.32 ± 0.29 (19)	9.80 ± 0.17 (25)
7.2 + vernalisation	12.79 ± 0.34 (19)	9.07 ± 0.35 (15)
13.1 + vernalisation	13.36 ± 0.33 (23)	9.79 ± 0.22 (24)
C24 + GA	19.10 ± 0.65 (20)	n.d.[e]
6.2 + GA	16.86 ± 0.40 (21)[d]	n.d.
7.2 + GA	15.90 ± 0.38 (20)[d]	n.d.
13.1 + GA	19.95 ± 0.64 (20)	n.d.

Table 3.3 Flowering time, expressed as total leaf number (TLN) of C24 wild type and three *METII* transgenic lines with reduced levels of *METII* expression. Experiment 1 was conducted in individual test tubes in a dedicated growth cabinet. Experiment 2 was conducted on large Petri plates in a general use growth room.

[a] average total leaf number at onset of flowering ± standard error (number of plants).

[b] significantly different to C24 at 5% level

[c] significantly different to C24 at 1% level

[d] significantly different to C24 + GA at 1% level

[e] not done

Regardless of the difference in flowering time of C24 between the two experiments, the behaviour of the *METII* lines followed exactly the same trend. In experiment 2, line 7.2 was the earliest flowering, with 15.5 leaves compared to 21.1 leaves of C24; line 6.2 flowered next, with 18.5 leaves; and line 13.1 flowered at almost exactly the same time as C24, after producing 21.2 leaves (Table 3.3, Experiment 2). Therefore even in conditions where C24 flowers quite early, two of the *METII* lines flower earlier than C24, and the promotion again correlates with the degree of reduction in *METII* expression. Thus it appears that a 40 % reduction in *METII* expression (in line 13.1) is insufficient to promote flowering, whereas a 60 % or greater reduction in *METII* expression does promote flowering.

3.3.7.2 METII transgenic lines respond to vernalisation

In the C24 ecotype, a three week vernalisation treatment induces early flowering (Finnegan *et al.*, 1998). The promotion of flowering in *METI* antisense plants is equivalent to approximately 50 % of the promotion caused by vernalisation of C24; therefore *METI*-induced demethylation partially substitutes for the vernalisation response (Finnegan *et al.*, 1998).

The vernalisation response of the *METII* transgenic lines was tested in the same two conditions as described in section 3.3.7. In experiment 1, vernalised C24 flowered with 12.7 leaves, compared to 33 leaves of unvernalised C24 (Table 3.3). Each of the *METII* lines also responded to vernalisation by flowering with fewer leaves than the unvernalised *METII* plants (Table 3.3). The same trend was observed in experiment 2. Therefore, reducing the level of *METII* expression did not prevent a vernalisation response in these lines, and the flowering time of each vernalised *METII* line was not significantly different to that of vernalised C24 (Table 3.3). The extent to which different *METI* lines substitute for

vernalisation is correlated with the extent of demethylation (Finnegan *et al.*, 1998). In the experiments described in section 3.3.7.1, the promotion of flowering in the *METII* line 7.2 was equivalent to 25 % of the promotion caused by vernalisation in experiment 1. In experiment 2, where C24 was earlier flowering, the promotion of flowering in line 7.2 was equivalent to 52 % of the C24 vernalisation response. Therefore a reduction in *METII* expression can also partially substitute for the vernalisation response in C24.

3.3.7.3 METII transgenic lines respond to exogenous GA

The plant hormone gibberellic acid (GA) promotes flowering of *Arabidopsis* (Langridge, 1957) and plants with a deficiency in GA or GA-signalling flower late (Koornneef *et al.*, 1985). One possible explanation for a reduction in *METII* expression promoting flowering could be that a component of the GA pathway is upregulated in the *METII* transgenic plants. To investigate this possibility, the flowering time of the *METII* lines was compared to that of C24 in the presence and absence of exogenous GA.

The experiment was carried out using plants grown in individual test tubes. GA-treated C24 plants flowered with 19 leaves compared to 33 leaves of untreated C24 (Table 3.3). In the presence of exogenous GA, the three *METII* lines flowered early compared with their respective untreated lines (Table 3.3). The trend of flowering promotion in untreated *METII* lines was conserved in the GA-treated lines, in that the earliest flowering line, 7.2, was also the earliest after a GA treatment. Line 13.1, which had no promotion of flowering compared to the C24 control, flowered at the same time in the presence of GA as did GA-treated C24 plants.

The effect of GA and the *METII* constructs for lines 6.2 and 7.2 was additive, as GA-treated *METII* lines flowered earlier than GA-treated C24 plants (Table 3.3). This could mean that either *METII* and GA act in separate pathways, both of which contribute to the early flowering response, or that *METII* and GA act in a common pathway but that the early flowering response is not saturated by either GA or the effect of the *METII* constructs. The GA treatment did not saturate the early flowering response, as vernalised C24 plants flowered with 12.68 leaves and GA-treated C24 flowered with 19 leaves (Table 3.3, Experiment 1). This is consistent with published reports of GA only partially substituting for the vernalisation response (Chandler and Dean, 1994).

3.3.8 Early flowering of *METII* transgenic plants is not mediated by an obvious reduction in *FLC* expression

The results described so far demonstrate that even though no detectable decrease in methylation occurs in *METII* transgenic lines, plants with at least a 60 % reduction in *METII* expression flower significantly earlier than wild type. In *METI* antisense (AMT) plants, the early flowering response has been attributed to a decrease in expression of the flowering repressor *FLC* (Sheldon *et al.*, 1999). The expression of *FLC* was therefore investigated in the *METII* transgenic lines, to see if their early flowering phenotype could also be explained by an effect on *FLC* expression.

RNA from C24 and the three *METII* transgenic lines was subjected to northern analysis using *FLC* as a probe. For lines 6.2, 7.2, and 13.1, no obvious decrease in *FLC* expression was observed in comparison to C24 (Figure 3.15a). In C24, a 7 day vernalisation treatment promoted flowering by 40 % compared to unvernalised plants, but the decrease in *FLC* expression, as judged by Northern analysis, was minimal (Sheldon et al., 2000). As

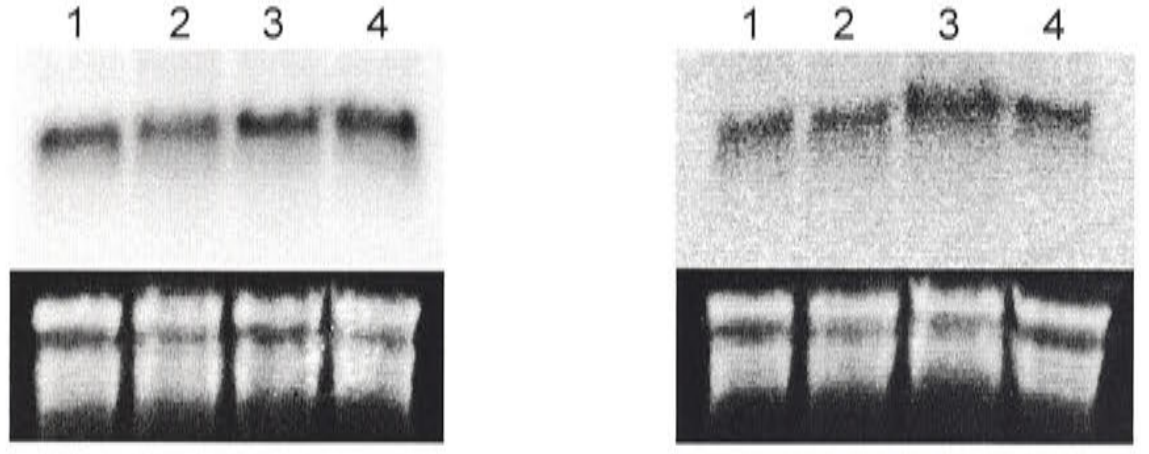


Figure 3.15 Expression of *FLC* and *SOC1* in C24 and three *METII* transgenic lines with reduced levels of *METII* expression. RNA was extracted from 19 day old seedlings, separated on 1.2% formaldehyde agarose gels and stained with EtBr. RNA was transferred to nylon filters and hybridised to either **(A)** *FLC* or **(B)** *SOC1* riboprobes. The EtBr-stained gels are shown below as loading controls. Lane 1, C24; lane 2, line 6.2; lane 3, line 7.2; lane 4, line 13.1.

the *METII* transgenic plants had at most a 25 % promotion of flowering compared to C24 in the absence of vernalisation (Table 3.3, experiment 2), it would be extremely difficult to see a corresponding decrease in *FLC* expression by northern analysis.

The *Suppressor of overexpression of CO 1* (*SOC1)* gene encodes a floral promoter downstream of and down-regulated by *FLC* (Samach *et al.*, 2000). Theoretically, if *FLC* expression was compromised in the *METII* lines, an increase in *SOC1* expression should be observed. The level of *SOC1* expression was therefore analysed in the three *METII* transgenic lines with reduced levels of *METII* expression. Consistent with the lack of noticeable *FLC* expression, there was no major increase in *SOC1* expression relative to C24 observed for any of the *METII* lines (Figure 3.15b).

3.3.9 Microarray analysis of a *METII* transgenic line

METII transgenic lines with a 60 % or greater reduction in *METII* expression flower early. The experiments described in the previous sections did not provide any explanation for this early flowering phenotype. To further investigate the molecular basis of the early flowering phenotype of the *METII* lines, the expression pattern of approximately 13,000 cDNA clones, representing about half of the *Arabidopsis thaliana* genome, was compared in C24 and the *METII* transgenic line 7.2 using microarray analysis. Line 7.2 was chosen for initial analysis as it has only 22 % of wild type levels of *METII* expression (Figure 3.7). T3 seed from the segregating T2 generation of line 7.2 was grown in the presence of kanamycin and RNA isolated from pooled kanamycin-resistant seedlings was used for the microarray analysis. The comparison and data analysis was carried out in quadruplicate as described in section 3.2.8.

The list of genes that were differentially induced or repressed by at least a factor of 2.0 in the *METII* line 7.2 as compared to C24 is summarised in Table 3.4. In total, 44 genes were up-regulated and 14 genes were down-regulated in line 7.2 compared to C24. Of the up-regulated genes, approximately 50 % had a role in photosynthesis; of these genes, 30 % were chloroplast encoded, and the remaining were nuclear-encoded. The photosynthetic genes encoded Rubisco subunits, components of photosystems I and II, chlorophyll binding proteins and light-harvesting chlorophyll proteins (see Appendix 1). This result suggests that photosynthesis may be affected in the *METII* transgenic line.

Nineteen of the up-regulated genes were either unnamed cDNA clones or unknown, putative or hypothetical proteins. Blast-n searches showed that three of these genes were also homologous to photosynthetic genes (Table 3.4). Other genes up-regulated in line 7.2 included *CHALCONE SYNTHASE* (*CHS*), *VEGETATIVE STORAGE PROTEIN* (*VSP*), peroxidase, catalase and ubiquitin-conjugating protein. These genes have roles in a variety of cell functions including photorespiration, lignification, flavinoid biosynthesis and cell maintenance (Appendix 1). The expression of *CHS*, *VSP* and catalase is light-regulated. Of the 14 genes which were down-regulated in line 7.2, most were unknown or putative proteins with no known function. The few genes with known function included a nodulin-like protein, a water stress-induced protein and an amine oxidase-like protein (Appendix 1).

To ensure that the relative changes in expression observed were not microarray artefacts, the expression profiles of several negative control genes were analysed. The expression levels of negative control genes such as beta-glucuronidase (uidA), globin and green fluorescent protein in this microarray experiment were at background level (results not shown). Thirty DNA elements have been identified that showed no major change in

Table 3.4 Expression profile of genes, grouped by function, that were differentially induced (>2.00, in black print) or repressed (<0.50, in blue print) in the *METII* transgenic line 7.2 compared to wild type C24. See also Appendix 1 for functions of known genes. N/A; no accession number available.

[a] median ratio of expression; where more than one value is given for the same gene, multiple clones were present on the slide.

[b] for unidentified cDNA clones, hypothetical and putative proteins, Blast-n searches of each sequence were performed and the closest match listed.

[c] chloroplast encoded gene

Gene annotation	Ratio[a]	Genepix ID #	Accession#	Clone ID#	Blast-N closest match[b]
Genes with photosynthetic roles					
Chlorophyll a/b binding protein 1 precursor	2.32	140	At2g34430	m6kp2d08f1	
Chlorophyll a/b binding protein 2 precursor	2.18	4327	At1g29930	m6kp50a07f1	
Chlorophyll a/b binding protein 4 precursor	2.37	4095	At3g47470	m6kp47f03f1	
Lhcb6 Light harvesting chlorophyll a/b protein	2.02	5894	AY062113	23A1T7	
Phosphate/triose phosphate translocator precursor	2.39	2559	At5g46110	m6kp28f03f1	
Photosystem I chain XI precursor[c]	2.08	4222	At4g12800	m6kp48h10f1	
Photosystem I subunit II precursor[c]	2.09	3096	At1g03130	m6kp36b12f1	
Photosystem I subunit V precursor[c]	2.34	3004	At1g55670	m6kp35c04f1	
Photosystem II 10kDa polypeptide precursor[c]	2.14	2485	At1g79040	m6kp27h01f1	
Photosystem II core complex protein Psb[c]	2.07 2.00	3144 2076	At1g67740 At1g67740	m6kp36f12f1 m6kp23e12f1	
Photosystem II D2 protein PsbD[c]	2.26	6158	At2g07738	slW122	
Putative chlorophyll a/b binding protein	2.15	3964	At2g05070	m6kp46c04f1	
Rotamase TLR40 precursor, thylakoid lumen	2.28	1026	At3g01480	m6lp11f06f1	

Gene annotation	Ratio[a]	Genepix ID #	Accession#	Clone ID#	Blast-N closest match[b]
Rubisco small unit	2.09	4893	At1g67090	m6kp55h90f1	
Rubisco small subunit 1b	2.52	3720	At5g38430	m6kp43f12f1	
Rubisco small subunit 2b	2.68	1980	At5g38420	m6kp22e12f1	
Rubisco small subunit 3b	2.91	4608	At5g38410	m6kp52g12f1	
	2.85	4913	At5g38410	m6kp56b05f1	
	2.55	3882	At5g38410	m6kp45d06f1	
	2.22	3830	At5g38410	m6kp44h02f1	
	2.62	2446	At5g38410	m6kp27d10f1	
Genes with other roles					
Amine oxidase-like protein	0.44	2341	At3g43670	m6kp26d01f1	
Catalase	2.04	7254	N/A	CA05e06	
Chalcone synthase	2.40	6819	At5g13930	177N23T7	
Nodulin-like protein	0.49	19763	At2g23990	T29E15.19	
Npr1-like-2	0.44	6363	N/A	N/A	
Probable major latex protein	2.28	4510	At4g23670	m6kp51h10f1	
Putative SF-16 protein (Helianthus annus)	2.44	3	At2g43680	m6kp1a03f1	
Prxr1 peroxidase	2.31	472	At4g21960	m6kp5h04f1	
	2.02	6029	N/A	slW411	
Putative peroxidase	0.46	20110	At2g34060	T14G11.18	
Ribosomal protein L32[c]	2.35	1345	P42354	m6kp16a01f1	
TNP2-like transposon protein	0.49	19524	N/A	T19K21.8	
Ubiquitin-conjugating protein	2.02	2388	At1g64230	m6kp26g12f1	
Vsp2 Vegetative storage protein	2.53	6248	At5g24770	slW212	
	2.36	1755	At5g24770	m6kp20c03f1	
Water stress-induced protein	0.49	2901	At1g54410	m6kp34b09f1	
	0.36	2342	At1g54410	m6kp26d02f1	

Gene annotation	Ratio[a]	Genepix ID #	Accession#	Clone ID#	Blast-N closest match[b]
Genes with unknown roles					
cDNA clone	2.14	17581	At1g19120	AA042151	Unknown
cDNA clone	4.15	17468	AA042459	H10E5T7	PSI P700 apoprotein A1
cDNA clone	2.07	17470	At5g17920	AA042464	Homocysteine S-methyltransferase
cDNA clone	2.13	17038	N96316	G8C7T7	Mouse RNA binding protein
cDNA clone	2.55	16800	N96461	F4C6T7	Rps7 ribosomal protein
cDNA clone	2.7	17420	W43884	H8A11T7	Part of chrom. 4
cDNA clone	2.11	17051	N96785	G9D1T7	Part of Arabidopsis cp genome
cDNA clone	2.31	15771	T42661	116C9T7	Similar to VIP2 protein
cDNA clone	2.16	16020	T76754	150P19T7	Ribosomal protein L32
cDNA clone	2.24	17229	At3g16130	W43642	PSI subunit XI precursor
cDNA clone	3.61	17421	W43885	H8A12T7	PSI P700 apoprotein A2
cDNA clone	2.1	7068	X71915	GBGA476	Epithiospecifier gene
cDNA clone	0.37	6192	AF446875	K18I23	Part of chrom. 5
cDNA clone	0.49	16869	N96600	F10F2T7	Part of chrom. 5
Hypothetical protein	2.01	19509	At2g10020	F7B19.16	
Hypothetical protein	2.12	17505	At3g11600	AA042189	Part of chrom. 3
Hypothetical protein	2.28	17187	At4g01150	N97218	Arabidopsis mRNA AF389292
Hypothetical protein	0.46	20198	At2g38680	T6A23.12	
Hypothetical protein SEB2	0.46	2591	At3g16860	m6kp28h11f1	Candidate ARA1 kinase
Putative protein	2.02	18825	At5g43980	MRH10_9	Unknown protein
Putative protein	0.40	16885	At4g26450	N96063	Part of chrom. 4
Putative protein	0.49	2330	At4g37300	m6kp26c02f1	Part of chrom. 4
Putative protein	0.31	18808	At5g56070	MDA7_12	Unknown
Unknown	2.22	6392	N/A	IW04e08	Part of chrom. 5
Unknown	2.22	309	N/A	m6kp4b09f1	
Unknown	2.43	1093	At1g78150	m6kp12d01f1	Part of BAC T11I11
Unknown	0.43	3013	N/A	m6kp35d01f1	

expression in 162 *Arabidopsis* microarray experiments, and thus serve as suitable reference genes (Wu *et al.*, 2001). Of these elements, eight were present on this microarray, and the median ratios of expression in the *METII* line and C24 of these elements ranged from 0.83 to 1.05. This indicates that the variability in expression of the other genes observed in the experiment reported here are likely to be real changes.

3.4 Discussion

Introduction of antisense and sense constructs against the *METII* gene into *Arabidopsis* caused a reduction in *METII* expression in three independent lines. Expression of *METII* was reduced by up to 80 % compared to wild type levels. Despite the decrease in *METII* expression, no overall reduction in genomic methylation levels was detected by RP-HPLC. Likewise, methylation-sensitive restriction enzyme assays did not detect any decrease in methylation at either CG and CTG sites within repetitive DNA sequences. In contrast, introduction of an antisense against the methyltransferase *METI* results in up to a 90 % decrease in methylation at CG sites (Finnegan *et al.*, 1996). However, it is possible that METII targets sequences other than CG, as similarity between *METI* and *METII* is lower in the target recognition domain, which is responsible for determining the sequence to be methylated, than it is in other conserved amino acid motifs (Genger *et al.*, 1999). It is also possible that METII methylates cytosines only in highly specific sequence contexts that are not detected by the assays used in this study. The human Dnmt3b enzyme selectively methylates certain satellite DNA sequences, establishing a precedent for sequence-specific methylation (Xu *et al.*, 1999).

Whereas *METI* antisense plants have a pleiotropic phenotype, displaying many developmental abnormalities (Finnegan *et al.*, 1996), the *METII* transgenic lines did not

show any morphological abnormalities. Similarly, mice with disrupted *Dnmt2* expression are phenotypically normal (Okano *et al.,* 1998b) and antisense *CMT3 Arabidopsis* plants have no morphological phenotype, even though they display reduced CNG methylation (Lindroth *et al.,* 2001). *ddm1* mutants also show no abnormalities in early generations, but do after repeated selfing (Kakutani *et al.,* 1995, 1996); perhaps the same could be true for the *METII* lines.

METII cannot substitute for *METI,* as *METII* is still expressed in *METI* antisense plants where CG methylation is reduced by up to 90 % (Genger *et al.,* 1999). Consistent with this, crossing lines with reduced levels *METII* expression to *fis* mutant plants does not rescue the *fis* mutant phenotype, whereas as crossing *METI* antisense plants to *fis* mutants does rescue the phenotype (Luo *et al.,* 2000). This indicates that METII does not appear to play the same role as METI in regulating the activity of seed development genes downstream of the *FIS* genes. Together, these results suggest that METII is likely to have completely different target sequences to METI.

Although methylation levels are not detectably reduced in the *METII* transgenic lines, decreasing *METII* expression by at least 60 % promotes flowering compared to wild type plants. The decrease in *METII* expression correlates with the time to flower, as the line with the greatest reduction in *METII* expression is the earliest flowering. In contrast, a line with a 40 % reduction in *METII* expression flowered at the same time as wild type plants. Perhaps a critical number of sites must be demethylated for promotion of flowering to occur, and the line with 40 % reduction in *METII* expression still has enough residual METII activity to keep those sites methylated.

Flowering is promoted by approximately 33 % in a *METI* antisense line that has an 80 % reduction in CG methylation levels (Finnegan *et al.*, 1998). In the *METII* cosuppressed line 7.2, flowering is promoted by 25 % (Table 3.3, Experiment 2). This line has an 80 % decrease in *METII* expression, but in contrast to the *METI* antisense line, the *METII* line has no detectable decrease in methylation. If the promotion of flowering in the *METII* transgenic plants is a result of the decrease in *METII* expression, this indicates that METII might methylate highly specific sites that are important for flowering.

AMT-induced demethylation partially substitutes for a vernalisation treatment in C24 plants by down-regulating *FLC* expression (Finnegan *et al.,* 1998; Sheldon *et al.*, 1999). A reduction in *METII* expression also promotes flowering, but has no detectable effect on *FLC* expression; therefore, the role of *METII* in the vernalisation response is unclear. Vernalisation results in a 15 % decrease in methylation levels in *Arabidopsis* (Finnegan *et al.,* 1998). Perhaps some of these same sites are normally methylated by METII, allowing for a small early flowering response in plants with reduced *METII* expression.

Microarray analysis revealed that the majority of genes upregulated in the *METII* transgenic line 7.2 are involved in photosynthesis. This suggests that METII might regulate or affect the transcription of a upstream gene that then affects transcription of photosynthetic genes. The upstream control points of photosynthesis are varied and include light, sugar signalling and hormone signalling (Smeekens, 2000). No key transcription factor that regulates the expression of many photosynthetic genes has been identified (R Furbank, personal communication), making a candidate for regulation by METII difficult to predict. It must also be considered that the *METII* transgenic plants could be at a different stage of development than the wild type plants, and that the upregulation of photosynthesis genes and early flowering could be an indirect result of an unrelated

effect, such as the shortening of an earlier growth phase. The expression of many photosynthetic genes varies with development, for instance, during the transition of tissues such as developing seeds and young leaves from source to sink organs as the plant matures (R Furbank, personal communication; Graham and Martin, 2000). Expression of photosynthetic genes also declines with age (J Evans, personal communication).

Genes related to photosynthesis are often among the most variable on microarray experiments, which is usually related to comparisons that have been made either between green and etiolated tissues, or between samples harvested at different times of the day (Wu *et al.*, 2001). This does not apply to the experiments reported here, as the plants were grown under identical light conditions and harvested within a few minutes of each other.

The up-regulation of photosynthetic genes provides a possible mechanism to explain the early flowering phenotype of the *METII* transgenic line, although photosynthetic rates would need to be measured in the *METII* transgenic plants and wild type plants to confirm this. An increased rate of photosynthesis would lead to a higher level of photosynthates, and photosynthate/carbohydrate supply is a determinant of flowering time. For example, exogenously-supplied sucrose induces flowering (Roldan *et al.,* 1999), and analysis of carbohydrate metabolic mutants such as *pgm* and *sex1* suggests that the availability of carbohydrate assimilates acts as a floral initiation signal (reviewed in Bernier *et al.,* 1993). In this respect it was interesting that a phosphate translocator gene (gene #At5g46110) was up-regulated in the *METII* transgenic line, as this protein is involved in regulating phosphate availability for sucrose synthesis (Taiz and Zeiger, 1991).

Approximately 30 % of the up-regulated photosynthetic genes were chloroplast encoded. Although there is some evidence for chloroplast DNA methylation (Ohta *et al.,* 1991;

Simkova, 1998), METII cannot be directly involved in the regulation of these genes, as it is predicted to be a nuclear protein (Genger, 2000) and does not contain a chloroplast localisation sequence (as determined by the "ChloroP" prediction program; Emanuelsson *et al.*, 1999). Therefore the up-regulation of the chloroplast encoded genes in the *METII* transgenic line is likely to be due to an effect on a nuclear-encoded upstream regulator of these genes. Understanding of nuclear-chloroplast signalling is still at an early stage, but it is known that nuclear genes can coordinate chloroplast gene expression, and that light-induced development, mediated by photoreceptors, involves the rapid accumulation of chlorophyll, photosynthetic membranes and photosynthetic proteins (Somanchi and Mayfield, 1999). The expression of two known genes, *DET* and *COP*, that repress transcription of genes required for chloroplast biogenesis (Deng *et al.*, 1992; Christopher and Hoffer, 1998) is not affected in the *METII* line, but there may be other as yet unknown genes involved in this process.

As well as photosynthetic genes, several other light-regulated genes, such as *CHALCONE SYNTHASE*, *VEGETATIVE STORAGE PROTEIN 2 (VSP2)* and catalase were up-regulated in the *METII* transgenic line. Interestingly, the *VSP2* gene is also upregulated in *METI* antisense plants, vernalised C24 plants, and *flc-20* mutants that have no FLC protein (C Helliwell, personal communication), raising the possibility that *VSP2* may be repressed by FLC. VSP1, a VSP2-related protein, has been shown to interact with the floral homeotic gene *AGAMOUS* and in addition to its role as a storage protein, is possibly involved in post-transcriptional regulation of transcription factors (Gamboa *et al.*, 2001), so may be involved in promoting flowering or floral development. Perhaps *METII* has a role in the regulation of *VSP2*.

Not all of the genes that were upregulated in the *METII* line are light-regulated. This suggests that the upregulation of gene expression in the *METII* transgenic line was caused by the *METII* construct, due either to decrease in *METII* expression or to an insertion effect of the transgene, and not due to experimental factors such as light conditions when harvesting. Of the few genes which were down-regulated in the *METII* transgenic line (see Appendix 1), no clear pattern emerged among them to explain the early flowering phenotype of this line.

Chapter 4: Epigenetic control of flowering

4.1 Introduction

A widespread loss of methylation can have variable and often unpredictable effects on gene expression, resulting in both silencing of normally expressed genes and activation of normally silenced genes (Li *et al.,* 1993). Within a methylation-depleted genome, genes can also become densely hypermethylated, resulting in dysregulation of gene expression. Both ectopic expression of genes in tissues where they are normally inactive (Kishimoto *et al.,* 2001) and silencing of normally active genes (Jacobsen and Meyerowitz, 1997) have been associated with hypermethylation.

In the *Arabidopsis* C24 ecotype, reducing CG methylation to 10 % of wild type levels via the introduction of an antisense construct against the methyltransferase *METI* (**a**ntisense **m**ethyl**t**ransferase, AMT) results in a wide range of developmental abnormalities, including reduced apical dominance and leaf size, decreased fertility and abnormal floral phenotypes (Finnegan *et al.,* 1996). Expression of a different AMT construct, covering a larger region of *METI,* in the Columbia (Col) ecotype yielded plants with approximately 30 % of wild type genomic methylation levels and causes a similar range of developmental abnormalities (Ronemus *et al.*, 1996). However, the effects of demethylation on flowering time of the two ecotypes are markedly different. Demethylation in C24 results in plants that flower earlier than wild type (Finnegan *et al.,* 1998), whereas demethylation in Col has the opposite effect, resulting in delayed flowering (Ronemus *et al.,* 1996). A second class of Col plants, obtained by an EMS-induced mutation in the *DDM1* (**d**ecreased **D**NA

methylation) gene, have 25-30 % of wild type levels of methylation (Vongs *et al.,* 1993). Unlike the C24-AMT and Col-AMT plants, *ddm1* plants do not initially exhibit any abnormal phenotypes, other than a slight delay in flowering (Vongs *et al.,* 1993; Kakutani *et al.,* 1995). However, after repeated selfing of the *ddm1* mutant, a pronounced delay in flowering time is observed along with other developmental abnormalities (Kakutani *et al.*, 1996; Kakutani, 1997).

The observations of the effect of AMT in Col and *ddm1* in Col raise two questions. Why does AMT in Col cause late flowering to occur immediately, but *ddm1* in Col only causes late flowering after several generations of inbreeding; and what is the mechanism by which a loss of methylation alters flowering time?

Given that demethylation by expression of AMT causes late flowering in Col and early flowering in C24, it is probable that the genotype of the ecotype is the major determinant of the response to demethylation. C24 and Col also have ecotype-specific responses to vernalisation. Many ecotypes of *Arabidopsis* flower early in response to vernalisation (Napp-Zinn, 1985); in long day (LD) conditions, vernalisation induces an early flowering response in C24 (Finnegan *et al.,* 1998), but not in Col (Lee and Amasino, 1995). This variation in vernalisation response between the two ecotypes has been attributed to allelic differences at *FRIGIDA (FRI)* and *FLOWERING LOCUS C* (*FLC),* two loci involved in the transition to flowering. *FRI* was first identified as a dominant gene conferring late flowering in the San-Feliu-2 (Sf2) ecotype, the repressive effect of which could be overcome by vernalisation (Lee *et al.,* 1993). Like Sf2, C24 has a dominant allele of *FRI* that causes late flowering which is reversible by vernalisation (Sanda and Amasino, 1995), whereas Col has a recessive *FRI* allele containing a deletion that prematurely terminates the open reading frame (Johanson *et al.*, 2000). *FLC* was identified as a locus that suppressed the late flowering caused by the *FRI*-Sf2 allele in the early flowering Landsberg *erecta* (Ler)

ecotype; when the *FLC* allele of Sf2 was combined with *FRI*-Sf2 in the Ler background, extremely late flowering was observed (Lee *et al.*, 1994). Like *FRI, FLC* is a repressor of flowering. Flowering time correlates with the level of *FLC* expression; ecotypes with high levels of *FLC* expression flower late (Sheldon *et al.*, 1999), whereas ecotypes with null alleles of *FLC* flower very early (Michaels and Amasino, 1999a). As *FRI* acts to upregulate *FLC*, the level of *FLC* expression depends on the nature of the *FRI* allele (Michaels and Amasino, 1999a; Sheldon *et al.*, 1999). C24, with a dominant *FRI* allele, has a moderately high level of *FLC* expression and flowers later than Ler and Col, which have recessive *FRI* alleles and very low levels of *FLC* expression (Sheldon *et al.*, 1999). However, the Col *FLC* allele does delay flowering when it is introgressed into the Ler background (Lee and Amasino, 1995; Sheldon *et al.*, 1999). *FLC* expression is down-regulated by both vernalisation (Michaels and Amasino, 1999a; Sheldon *et al.*, 1999) and demethylation (Sheldon *et al.*, 1999). The mechanism by which demethylation down-regulates *FLC* expression is unknown, although it does not involve changes in the methylation status of the *FLC* sequence (EJ Finnegan, personal communication). The different levels of *FLC* expression observed in C24 and Col provide an explanation for why C24 responds to vernalisation and demethylation by flowering early, but Col does not. Reducing the *FLC* expression of Col further by demethylation or vernalisation has no effect on flowering time, as the level of *FLC* expression in Col is already low (Sheldon *et al.*, 1999).

There are distinct differences between AMT and *ddm1* both in the way they affect methylation levels and the sequences that are affected. The *METI* antisense, which has a dominant effect, causes a reduction in methylation due to loss of methyltransferase activity (Finnegan *et al.*, 1996). In contrast, levels of methyltransferase activity are not depleted in the recessive *ddm1* mutants (Kakutani *et al.*, 1995). The *DDM1* gene product has homology to the SWI/SNF group of chromatin-remodelling ATPase proteins which modify

or disrupt protein-DNA interactions (Jeddeloh *et al.,* 1999; Brzeski and Jerzmanowski, 2003). It is thought to act as a transcriptional co-activator, or by facilitating the methylation of certain sequences, perhaps by physically allowing access of methyltransferases to DNA (Jeddeloh *et al.,* 1999). In the last case, when *DDM1* is mutated, DNA sequences would no longer be accessible to methyltransferases, and the level of methylation would consequently decrease.

In *METI* antisense plants loss of methylation occurs mostly within symmetric CG sequences, in both repeated sequences such as centromeric and ribosomal DNA in plants from the T1 and all subsequent generations (EJ Finnegan, personal communication; Finnegan *et al.,* 1996; Kishimoto *et al.*, 2001), as well as in at least four single-copy sequences in T2 generation plants (Ronemus *et al.,* 1996). In *ddm1* mutants decreased methylation at both CG and CNG sites is observed, initially only in repeated sequences such as ribosomal DNA genes; in early generations of *ddm1,* two single-copy sequences that are normally methylated in wild type plants remain methylated (Vongs *et al.,* 1993). However, after six generations of inbreeding, loss of methylation at these single-copy sequences is observed, in conjunction with the accumulation of developmental abnormalities and late flowering (Kakutani *et al.,* 1996; Kakutani, 1997). Therefore in AMT and *ddm1* plants, a loss of methylation occurs at different rates and at different sites, reflecting the different mechanisms causing demethylation.

The late flowering phenotype of inbred *ddm1* plants was mapped to a region containing the flowering time gene *FWA* (Kakutani, 1997). *FWA* is a repressor of flowering, first identified in the late flowering semi-dominant *fwa* mutant (Koornneef *et al.,* 1991). The Ler wild type does not express *FWA,* as the promoter is methylated, but hypomethylation within a 5 Mb region around *FWA* results in its expression in the *fwa* mutant (Soppe *et al.,*

2000). Repeated selfing of the *ddm1* mutant, which results in a loss of methylation at single-copy sequences, is associated with demethylation of *FWA* and late flowering (Soppe *et al.*, 2000).

The experiments described in this chapter attempted to elucidate the molecular basis for the different effects of demethylation on flowering time in the C24 and Col ecotypes. At the time the backcrossing program described in this chapter commenced, it was not known that the up-regulation of *FWA* causes late flowering in Col plants with low methylation and the down-regulation of *FLC* causes early flowering in C24 plants with low methylation. The experiments also confirmed that both AMT and *ddm1* in the Col background delay flowering, but that this delay occurs in different generations, reflecting the different mechanisms of demethylation. Could the fact that AMT immediately affects both repeated and single copy sequences, whereas *ddm1* initially affects repeated sequences, and single copy sequences only after several generations of inbreeding, contribute to these opposing effects? The effects of AMT and *ddm1*-induced demethylation were compared in genetic backgrounds which had equivalent alleles of *FRI*, *FLC* and *FWA*. This enabled an investigation into whether like AMT, *ddm1* could promote flowering in a background with high levels of *FLC* expression, and into the mechanism by which *FLC* down-regulation occurs in response to demethylation.

4.2 Materials and Methods

4.2.1 Flowering time experiments

Flowering time experiments were carried out under LD conditions (16 h light, 8 h dark) in individual test tubes or in pots as per section 2.9. The light intensity ranged from 100-120 μE.

4.2.2 Plant lines used for backcrossing

The early-flowering third generation *METI* antisense line #10.5 (AMT, anti-methyltransferase) in the C24 background (Finnegan *et al.*, 1996) and the late-flowering seventh generation *ddm1* mutant line #10 in the Columbia background (gift of Eric Richards, Washington University, St Louis) were crossed to near-isogenic Landsberg *erecta* (Ler) lines into which the *FLC* allele of either Col (Ler *FLC*-Col; Koornneef *et al.*, 1994) or Sf2 (Ler *FLC*-Sf2; Lee *et al.*, 1994) had previously been introgressed. C24 (AMT wild type) and Col (*DDM1* wild type) were crossed to the two Ler lines in parallel as controls for the genetic backgrounds of AMT and *ddm1*.

4.2.3 Screening F1 progeny

All F1 progeny were screened using the *nga III* SSLP microsatellite marker. PCR reactions contained 10 mM Tris pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 200 μM each of dATP, dTTP, dGTP and dCTP, 0.25 μM of each primer, 1 unit of Perkin-Elmer *Taq* polymerase and 1 μL of template DNA (prepared using a modification of the Edwards plant DNA mini-preparation, see section 2.5.3) in a final volume of 20 μL. Primers were CTC CAG TTG GAA GCT AAA GGG (*nga III*-F) and TGT TTT TTA GGA CAA ATG GCG (*nga III*-R) (Bell

and Ecker, 1994). Cycling conditions for the *nga III* PCR were as follows: one cycle of 94 °C 3 min, 50 °C 30 sec, 72 °C 30 sec; 29 cycles of 94 °C 10 sec, 50 °C 10 sec, 72 °C 30 sec; and a final cycle of 72 °C 10 min, 25 °C 2 min. Reaction products were resolved on 8 % acrylamide gels which were stained for 15 min in 50 μg/μL EtBr.

4.2.4 Generation and screening of backcrossed plants

Heterozygous F1 progeny identified by *nga III* SSLP analysis were backcrossed to the recurrent Ler parental line, followed by four additional backcrosses to produce near isogenic lines with normal and low levels of methylation. The methods used to screen each generation of backcrossed plants are described in the following sections.

4.2.4.1 AMT backcrosses

Backcrosses of AMT to Ler *FLC*-Sf2 and Ler *FLC*-Col were screened at each generation for the presence of the *METI* antisense transgene by PCR as per section 2.4.

4.2.4.2 ddm1 backcrosses

Backcrossed progeny of *ddm1* to Ler *FLC*-Sf2 and Ler *FLC*-Col were screened at each generation for the presence of the *m555* **c**leaved **a**mplified **p**olymorphic **s**equence (CAPS) marker. PCR conditions were as per section 4.2.3 except that 1.0 μM of each primer was used. Primers were CTC TTG AAT TAT ATT AAG TTG ACT AG (*m555*-1) and CCT TTA ATT AGT TAT CAA ATC (*m555*-2) (TAIR genetic marker accession #1945684; www.arabidopsis.org). Cycling conditions for the *m555* PCR were 30 cycles of 93 °C 30 sec, 49 °C 30 sec and 72 °C 30 sec. The PCR reaction products were purified on Sephadex G-50 spin columns and digested with *Acc*I (New England Biolabs) overnight in

1 x J universal restriction buffer (section 2.6.1) at 37 °C. Digests were resolved on 8 % acrylamide gels which were stained for 15 min in 50 μg/μL EtBr.

4.2.4.3 C24 and Col (DDM1) backcrosses

The F1 and BC1 progeny of the C24 and the Col crosses were screened using the *nga III* SSLP marker as per section 4.2.3. Subsequent generations were not screened.

4.2.5 Genotyping of BC5 lines

4.2.5.1 Genotyping at FLC

C24 and AMT lines backcrossed to Ler *FLC*-Sf2 were genotyped at the *FLC* locus using an *FLC* CAPS marker to differentiate between C24 and Sf2. PCR conditions were as for section 4.2.3 except that the primers used were GCA ATA GCG AGC AGT GGC GG (*JO011-P1*) and GCC GCA TTG TAG CTA CAT G (*JO015-P1*). Cycling conditions for the *FLC* CAPS PCR were as follows: one cycle of 93 °C 2 min, 60 °C 1 min, 72 °C 1 min; 30 cycles of 93 °C 30 sec, 60 °C 30 sec, 72 °C 30 sec; and a final cycle of 72 °C 5 min, 25 °C 2 min. The PCR reaction products were purified on Sephadex G-50 spin columns and digested with *Taal* (MBI Fermentas) overnight in 1 x J universal restriction buffer (section 2.6.1) at 37 °C. Reaction products were resolved on 8 % acrylamide gels which were stained for 15 min in 50 μg/μL EtBr.

C24 and AMT lines backcrossed to Ler *FLC*-Col were genotyped at the *FLC* locus by PCR using an *FLC* sequence polymorphism to differentiate between the C24 and Col ecotypes. The *FLC* PCR conditions were as per section 4.2.3 and sequencing was performed as per section 2.10 using the *JO015-P1* primer.

Col and *ddm1* lines backcrossed to Ler *FLC*-Sf2 were genotyped at the *FLC* locus using the *nga 225* SSLP marker to differentiate between Col and Sf2. PCR conditions were as for section 4.2.3 except that the primers used were GAA ATC CAA ATC CCA GAG AGG (*nga 225-F*) and TCT CCC CAC TAG TTT TGT GTC C (*nga 225-R*). Cycling conditions for the *nga 225* PCR were as follows: one cycle of 93 °C 2 min, 60 °C 1 min, 72 °C 1 min; 30 cycles of 93 °C 30 sec, 60 °C 30 sec, 72 °C 30 sec; and a final cycle of 72 °C 5 min, 25 °C 2 min. Reaction products were resolved on 2 % agarose gels and visualised with EtBr.

4.2.5.2 Genotyping at FRI

C24 and AMT lines backcrossed to Ler *FLC*-Sf2 and to Ler *FLC*-Col were genotyped at the *FRI* locus by PCR using an *FRI* sequence polymorphism to differentiate between C24 and Ler. The *FRI* PCR conditions were as per section 2.4, except that the primers used were ACA GAG TCT ACA AGT ATG G *(ERI-F)* and CTG GCA GAG CTC ATA GG *(ERI-R)*. Sequencing was performed as per section 2.10 using the *ERI-F* primer. Col and *ddm1* lines backcrossed to Ler *FLC*-Sf2 were genotyped at the *FRI* locus by PCR using an *FRI* sequence polymorphism to differentiate between Ler and Col *FRI* alleles in the same manner.

4.2.6 Selection of *ddm1* homozygous lines

Progeny plants of the selfed BC5 Ler *FLC*-Sf2/*ddm1* line were screened by PCR to identify plants homozygous for the *ddm1-1* mutation. PCR conditions were as per section 4.2.3, except that primers used were TCT CTC TCT TTG CCT TGA AAC A (*ddm1-1L)* and GTC CAT TTG AGG GTT CTG GA (*ddm1-1R)*. Cycling conditions for the *ddm1-1* PCR were as follows: one cycle of 93 °C 2 min, 55 °C 1 min, 72 °C 1 min; 30 cycles of 93 °C 30 sec, 55 °C 30 sec, 72 °C 30 sec; and a final cycle of 72 °C 5 min, 25 °C 2 min. Sequencing of

PCR products was performed as per section 2.10 using either the *ddm1-1L* or the *ddm1-1R* primer.

4.2.7 Methylation analysis

DNA from the backcrossed lines was assayed for CG methylation at repeated sequences by digestion with *Msp*I and *Hpa*II and hybridisation to a 180 bp centromeric repeat probe (Martinez-Zapater *et al.*, 1986) as per section 2.6.1. Southern analysis of the single-copy *FWA* gene was performed as above except that digested DNA was hybridised to the VE030 plasmid containing *FWA* cDNA #VBVAF04, corresponding to Genbank accession #Z30932 (W Soppe, personal communication).

4.2.8 RNA expression analysis

Total RNA was extracted as per section 2.7 and was assayed by Northern analysis for the level of *UFC, FLC* and *SOC1* expression as per section 2.8.1. The *UFC* riboprobe was prepared from the *Bam*HI-linearised PJO034 plasmid containing a 1.4 kb fragment covering the complete cDNA (Sheldon *et al.*, 1999). For details of *FLC* and *SOC1* riboprobes, see section 3.2.6.

4.2.9 RT-PCR for *FWA* expression

RT-PCR for *FWA* expression was performed using the Access RT-PCR system (Promega). RT-PCR reactions contained 1 x AMVRT/Tfl buffer, 1 mM $MgSO_4$, 0.2 mM dNTP's, 50 ng each primer, 1 unit of AMV reverse transcriptase, 2 units of *Tfl*I polymerase and 1 μg of DNase-treated RNA in a final volume of 20 μL. Primers for *FWA* were GCA AAT GGG TCA ACG TGT TTG C (*5' fwa-cod3*) and TCA GTC AAG TTG GTA GAT GAA

AG (*3' fwa-cod3*). Cycling conditions for the *FWA* RT-PCR were as follows: one cycle of 48 °C 45 min; 40 cycles of 95 °C 30 sec, 55 °C 30 sec, 72 °C 1 min; and a final cycle of 72 °C 5 min, 28 °C 1 min. Reaction products were resolved on 2 % agarose gels and visualised with EtBr.

4.3 Results

4.3.1 Demethylation in Columbia delays flowering

Plants of the C24 ecotype with reduced methylation levels flower earlier than wild type plants in LD (Finnegan *et al.*, 1998). Preliminary experiments in this laboratory confirmed that early generations of *ddm1* flowered only two days later than Col in LD (Genger, 2000). However, later generations of *ddm1* were reported as having a distinct late flowering phenotype (Kakutani, 1997) and as mentioned, expression of an antisense *METI* construct in Col also delayed flowering (Ronemus *et al.*, 1996). To confirm reports that demethylation in Col delays flowering, the flowering time of seventh-generation *ddm1* mutants and of Col plants carrying the same AMT construct that was originally used to transform C24 was measured in LD conditions.

In the first experiment, Col flowered after 19.1 days, whereas the *ddm1* mutant took 32.1 days to flower (Table 4.1). This agrees with published results of *ddm1* being late flowering (Kakutani, 1997). To confirm that the genetic background is an important determinant of flowering time, and that the difference was not just due to variable effects of *ddm1* and AMT, Col lines containing the AMT transgene (gift of Herve Vaucheret, INRA, France) were tested in the same conditions. Two lines, AMT-Col #46 and AMT-Col #95, which had been identified as having reduced levels of methylation (H Vaucheret, personal

Plant line	Flowering time (days)	
	Unvernalised	Vernalised
Columbia	19.1 ± 0.4	18.2 ± 0.3
ddm1	32.1 ± 1.0	33.9 + 1.1

Table 4.1 Flowering time of Columbia and *ddm1* in LD conditions expressed as number of days ± SE.

Plant line	Flowering time (days)
Columbia	13.9 ± 0.6
AMT-Col #46	32.3 ± 1.2
AMT-Col #95	29.6 + 0.4

Table 4.2 Flowering time of Columbia and AMT-Col lines in LD conditions, expressed as number of days ± SE.

communication) were analysed. In this experiment, Col flowered after 13.9 days (Table 4.2). Flowering was delayed in both AMT-Col lines; line #46 flowered after 32.3 days, and #95 flowered after 29.6 days (Table 4.2). Therefore when the AMT transgene is expressed in the Col background, late flowering is observed, suggesting that the genetic background is an important determinant of the flowering response to demethylation.

The genetic background of C24 and Col also determines their response to vernalisation. C24, with high *FLC* expression levels, flowers early in response to vernalisation in LD (Finnegan *et al.*, 1998) due to down-regulation of *FLC* expression (Sheldon *et al.*, 1999); Col, with low levels of *FLC* expression, is not responsive to vernalisation (Lee and Amasino, 1995). The *ddm1* mutant in the Col background also has low levels of *FLC* expression. To determine whether the late flowering *ddm1* mutant could respond to vernalisation, the flowering time of vernalised Col and *ddm1* mutants was measured in LD. A three week vernalisation treatment resulted in Col flowering after 18.2 days, compared to 19.1 days of unvernalised control plants (Table 4.1). Vernalised *ddm1* plants flowered after 33.9 days, compared to 32.1 days for unvernalised controls (Table 4.1). Therefore, late flowering Col plants with low methylation levels do not respond to vernalisation in LD. The late flowering phenotype of late-generation *ddm1* plants has been mapped to the *FWA* gene (Kakutani, 1997), with demethylation of *FWA* in *ddm1* plants resulting in its expression (Soppe *et al.*, 2000). The *fwa* mutant, which expresses *FWA*, does not respond to vernalisation (Koornneef *et al.*, 1991), consistent with *ddm1* lacking a vernalisation response.

Together, these results show that the genetic background of each ecotype clearly plays a part in determining the response of the ecotype to vernalisation and demethylation. AMT promotes flowering in backgrounds with high *FLC* levels, whereas *ddm1* delays flowering

in a low *FLC* background, due to demethylation and up-regulation of *FWA*. Could *ddm1* have the same effect as AMT and promote flowering in a background with high *FLC* expression?

The mechanism by which demethylation leads to the down-regulation of *FLC* expression is unknown. It does not involve direct demethylation of the *FLC* promoter or coding region (EJ Finnegan, personal communication), but may involve either demethylation of a trans-acting repressor of *FLC*, or an alteration of chromatin structure induced by low methylation, that could act as part of a long-range mechanism. As mentioned, AMT affects methylation of both single-copy and repeated sequences (Finnegan *et al.*, 1996; Ronemus *et al.*, 1996) whereas *ddm1* initially affects repeated sequences (Vongs *et al.*, 1993) and only affects single-copy sequences after repeated selfing (Kakutani *et al.*, 1996). The mechanism of *FLC* down-regulation could require demethylation of single copy sequences, or repeated sequences, or both.

4.3.2 Production of backcrossed lines

To investigate the role of demethylation of single copy vs repeated sequences in the down-regulation of *FLC*, AMT plants and *ddm1* mutant plants were crossed to Ler lines containing dominant alleles of *FLC* from either Col or Sf2 as per section 4.2.2. Both Ler lines have the recessive null Ler *FRI* allele (Johanson *et al.*, 2000), moderately high levels of *FLC* expression (Sheldon *et al.*, 1999) and the Ler *FWA* allele, which is not expressed (Soppe *et al.*, 2000). It should therefore be possible to observe the effect of demethylation caused by AMT and *ddm1* on an equivalent basis in these lines.

The F1 progeny of crosses between C24, AMT, Col or *ddm1* and the isogenic Ler lines were screened using the *nga III* SSLP microsatellite marker. The *nga III* primers flank simple sequence repeats in the *Arabidopsis* genome (Bell and Ecker, 1994) and the PCR amplification product distinguishes between Ler, C24 and Col in a co-dominant fashion, enabling heterozygotes to be easily identified. Figure 4.1 shows the PCR products amplified from three representative F1 heterozygous progeny plants of the C24 and AMT x Ler *FLC*-Col or Ler *FLC*-Sf2 crosses. Figure 4.2 shows the SSLP analysis of eight representative F1 heterozygous progeny plants of the Col x Ler *FLC*-Col or Ler *FLC*-Sf2 crosses.

The first and subsequent backcrosses (BC1 - BC5) of AMT to Ler *FLC*-Sf2 and Ler *FLC*-Col were screened for the presence of the *METI* antisense transgene by PCR. Figure 4.3 shows a representative sample of products amplified from DNA of the BC1 Ler *FLC*-Sf2/AMT plants. Plants #1-6, #8 and #9 (lanes 3-8, 10 and 11) were positive for the AMT transgene. Results for the BC2 to BC4 generations are not shown. Figure 4.4 shows the final PCR screening of the BC5 Ler *FLC*-Col/AMT and BC5 Ler *FLC*-Sf2/AMT lines. Plants that had inherited the transgene were identified as BC5 Ler *FLC*-Col/AMT #3 and #4 (lanes 5 and 6), and BC5 Ler *FLC*-Sf2/AMT #1 and #3 (lanes 9 and 11). Selfed plants of the AMT BC5 lines were not tested for the presence of the *METI* antisense transgene. The BC5 generation would have been heterozygous for the transgene, and the BC5⊗ population would be segregating for the transgene. However, as *METI* antisense plants that are null for the transgene still have low methylation levels and flower early (Finnegan *et al.*, 1998), the effect of the *METI* antisense should still be observed in this segregating population.

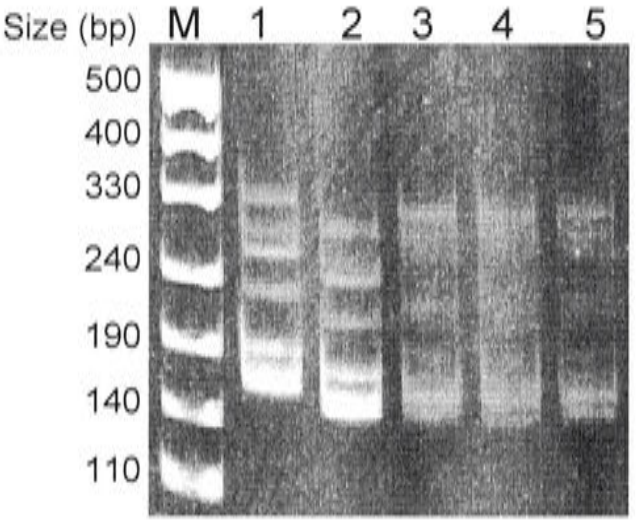


Figure 4.1 *Nga III* SSLP analysis of F1 plants on an 8% acrylamide gel.

Lane M, pUC19/*Hpa*II marker; lane 1, C24; lane 2, Ler; lane 3, AMT x Ler *FLC*-Col; lane 4, C24 x Ler *FLC*-Col; lane 5, C24 x Ler *FLC*-Sf2. Heterozygous plants (lanes 3-5) contain bands corresponding to those found in both the Ler parent and the C24 parent.

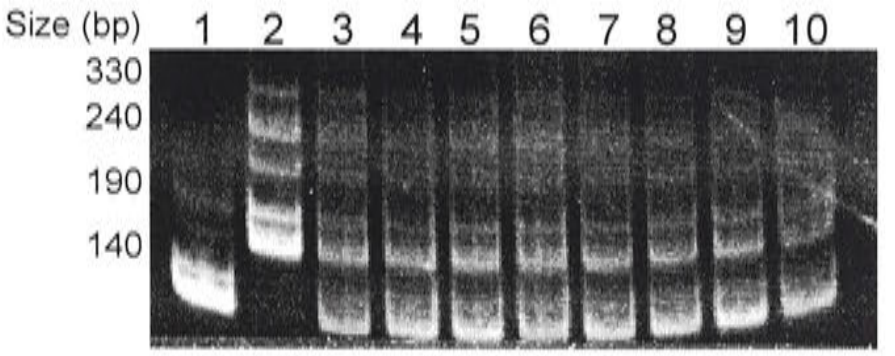


Figure 4.2 *Nga III* SSLP analysis of F1 plants on an 8% acrylamide gel.

Lane 1, Col; lane 2, Ler; lane 3 - 7, Col x Ler *FLC*-Sf2 #1-5; lane 8-10, Col x Ler *FLC*-Col #1-3. Heterozygous plants (lanes 3-10) contain bands corresponding to those found in both the Ler parent and the C24 parent.

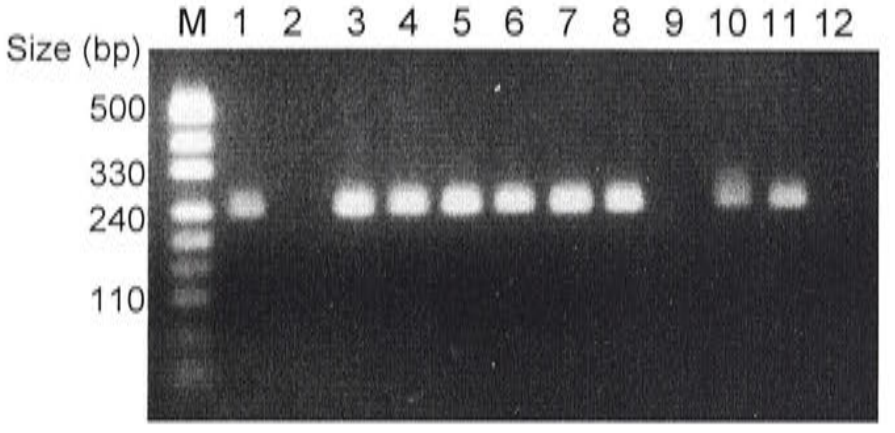


Figure 4.3 AMT PCR analysis of BC1 plants on a 2% agarose gel.

Lane M, pUC19/*Hpa*II marker; lane 1, AMT line 10.5 positive control; lane 2, water negative control; lanes 3 - 12, individual BC1 Ler *FLC*-Sf2/AMT plants #1-10.

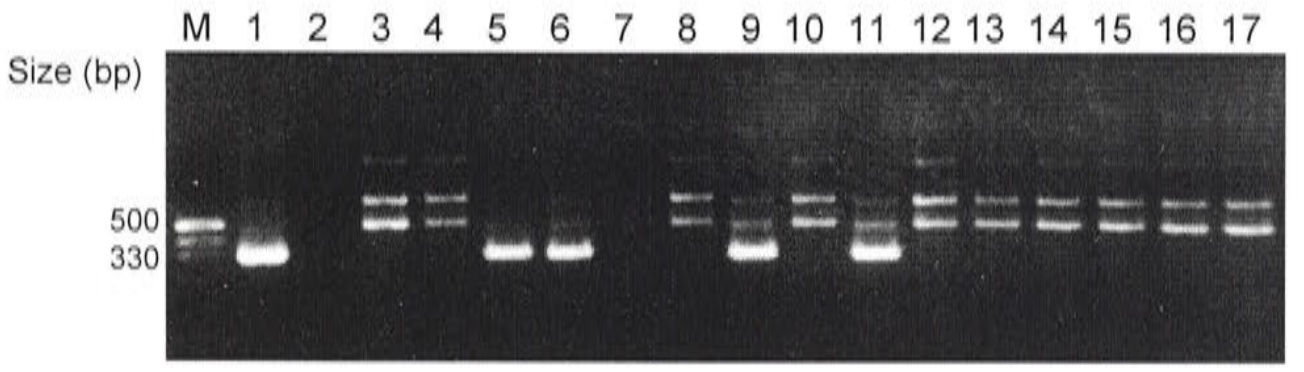


Figure 4.4 AMT PCR analysis of BC5 plants on a 2% agarose gel.

Lane M, marker pUC19/*Hpa*II; lane 1, AMT line 10.5 positive control; lane 2, water negative control; lanes 3 - 8, individual BC5 Ler *FLC*-Col/AMT plants #1-6; lanes 9-17, individual BC5 Ler *FLC*-Sf2/AMT plants #1-9. Bands at 500 bp and above are the result of non-specific amplification. Only plants with a strong band at 250 bp matching that of the positive control were classed as positive for the AMT construct.

The backcrosses of *ddm1* to Ler *FLC*-Sf2 and Ler *FLC*-Col were screened for the presence of the *m555* cleaved amplified polymorphic sequence (CAPS) marker to differentiate between Col and Ler ecotypes at the *m555* locus, which is closely linked to the *DDM1* locus on Arabidopsis chromosome V (*Arabidopsis thaliana* Science Genome Map 9; TAIR, *www.arabidopsis.org*). The 150 bp Col PCR product is not cut by *Acc*I, but the Ler product is cut once, producing fragments of 100 and 50 bp. Heterozygous plants should therefore have three bands, one each of 150, 100 and 50 bp, whose intensity decreases in proportion to their size. Figure 4.5 shows the CAPS analysis of a representative sample of BC1 Ler *FLC*-Sf2/*ddm1* plants. Plants #1 and #4 (lanes 4 and 7) were identified as heterozygotes and used in subsequent backcrossing. Subsequent Ler *FLC*-Sf2/*ddm1* BC generations were screened in the same manner (results not shown). Figure 4.6 shows the final CAPS analysis of the BC5 Ler *FLC*-Sf2/*ddm1* plants. Plants #1, #2 and #9 (lanes 4, 5 and 12) were heterozygous for the *m555* marker from Col.

When screening of the Ler *FLC*-Col/*ddm1* BC1 generation commenced, it became apparent that either no heterozygotes existed among the plants tested, or that the *m555* marker failed to distinguish between Ler *FLC*-Col/*ddm1* DNA and Col DNA (Figure 4.7). DNA from the Ler *FLC*-Col parental line was subsequently analysed using the *m555* CAPS PCR (Figure 4.8). The Ler *FLC*-Col PCR product (lane 4) was not cut by *Acc*1, producing a fragment indistinguishable from Col (lane 2). These data suggest that when *FLC*-Col was introgressed into the Ler background that either a large section of the Col chromosome containing the *m555* locus as well as *FLC* was introgressed, or that a double crossover between *m555* and *FLC* may have occurred. *FLC* and *DDM1* are at opposite ends of chromosome V (Figure 4.9). As the Ler *FLC*-Col/*ddm1* backcrossed lines could not be distinguished from the Ler *FLC*-Col parental line using the *m555* marker that is closely linked to *DDM1*, they were abandoned after the first BC generation, because

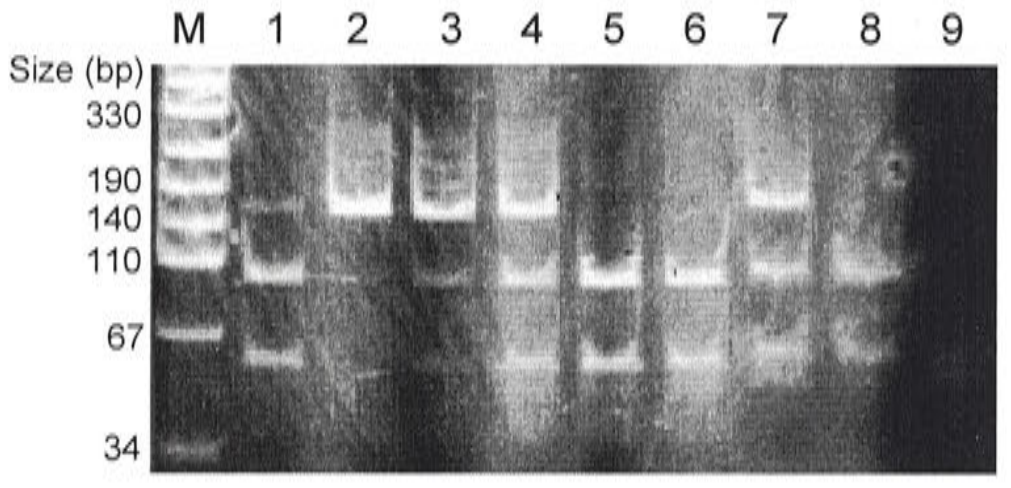


Figure 4.5 *m555* CAPS analysis of BC1 plants on an 8% acrylamide gel.

Lane M, pUC19/*Hpa*II marker; lane 1, Ler; lane 2, Col; lane 3, Ler + Col reconstructed heterozygote; lanes 4 - 8, individual BC1 *ddm1* x Ler *FLC*-Sf2 plants #1-5; lane 9, water negative control. Note: the digest of Ler is incomplete, hence a faint non-stoichiometric band is seen at 150 bp in lane 1. This incomplete digest can be differentiated from a heterozygote, e.g. in lane 4, because the lower bands in lane 1 are much brighter than the 150 bp band.

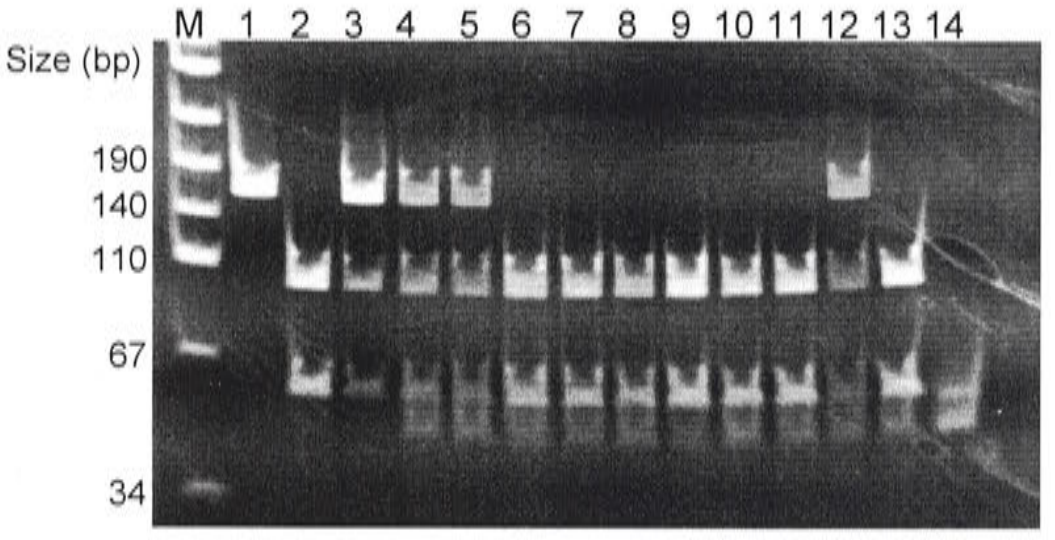


Figure 4.6 *m555* CAPS analysis of BC5 plants on an 8% acrylamide gel.

Lane M, pUC19/*Hpa*II marker; lane 1, Col; lane 2, Ler; lane 3, Ler + Col reconstructed heterozygote; lanes 4-13, individual BC5 Ler *FLC*-Sf2/*ddm1* plants #1-10; lane 14, water negative control containing primer-dimers.

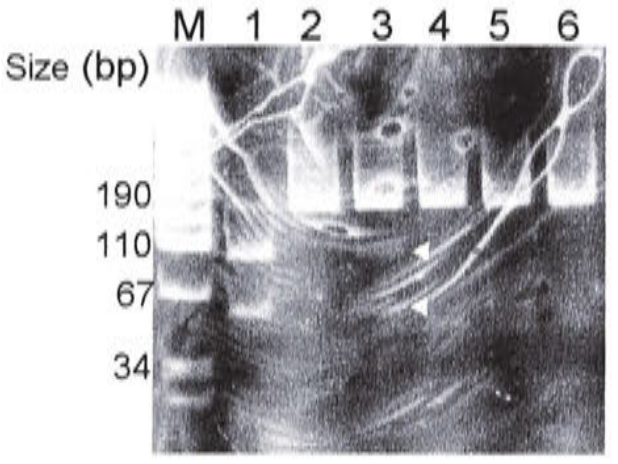


Figure 4.7 *m555* CAPS analysis of BC1 plants on 8% acrylamide gel.

Lane M, marker pUC19/HpaII; lane 1, Ler; lane 2, Col; lane 3, Ler + Col reconstructed heterozygote; lanes 4 - 6, individual BC1 Ler *FLC*-Col/*ddm1* plants #1-3.

Note: the 100bp and 50 bp Ler bands in lane 3 are very faint (white arrowheads), probably due to the reconstructed heterozygote sample containing less Ler DNA than Col DNA .

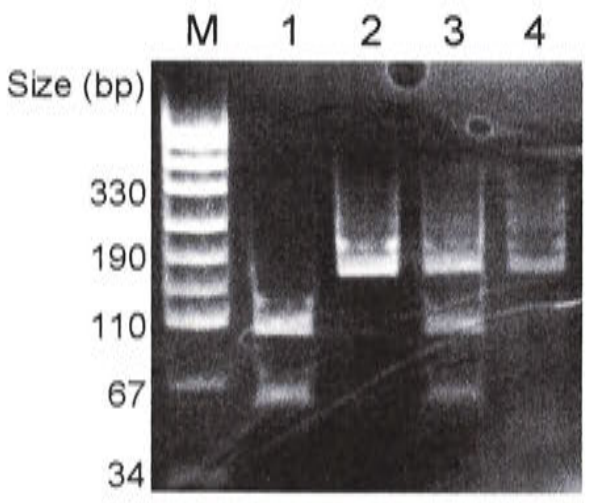


Figure 4.8 *m555* CAPS analysis of parental lines on an 8% acrylamide gel.

Lane M, marker pUC19/*Hpa*II; lane 1, Ler; lane 2, Col; lane 3, Ler + Col reconstructed heterozygote; lane 4, Ler *FLC*-Col parental line.

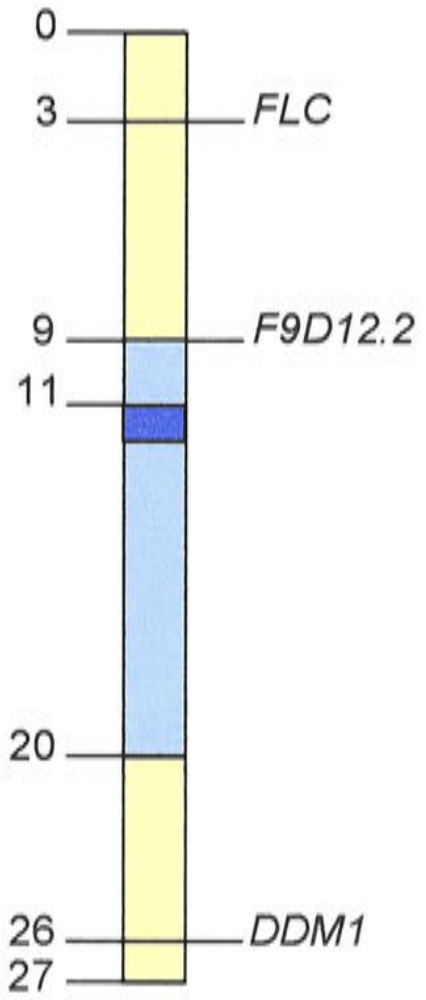


Figure 4.9 Chromosome V. Data taken from AGI map via *www.arabidopsis.org*

Dark blue region indicates centromere, light blue region indicates areas of highest transposon frequency (The Arabidopsis Genome Initiative, 2000). Scale is in megabases.

introgression of the *ddm1* mutation could not be assessed. Since this experiment commenced, *DDM1* and its various mutant alleles have been sequenced. If this information had been available at the time, then PCR and sequencing across the mutation site could have been used to identify the Ler *FLC*-Col/*ddm1* lines.

The BC1 progeny of the C24 x Ler *FLC*-Col, C24 x Ler *FLC*-Sf2, and Col x Ler *FLC-Sf2* crosses were screened using the *nga III* SSLP marker to ensure that the pollen donor had been the F1 heterozygote and not the Ler parental line (results not shown). However, the progeny of subsequent backcrosses were not screened for this marker in order to avoid selecting for either C24 or Col genomic sequences identified by the *nga III* primers, lest these affect flowering time.

4.3.3 Genotyping of backcrossed lines

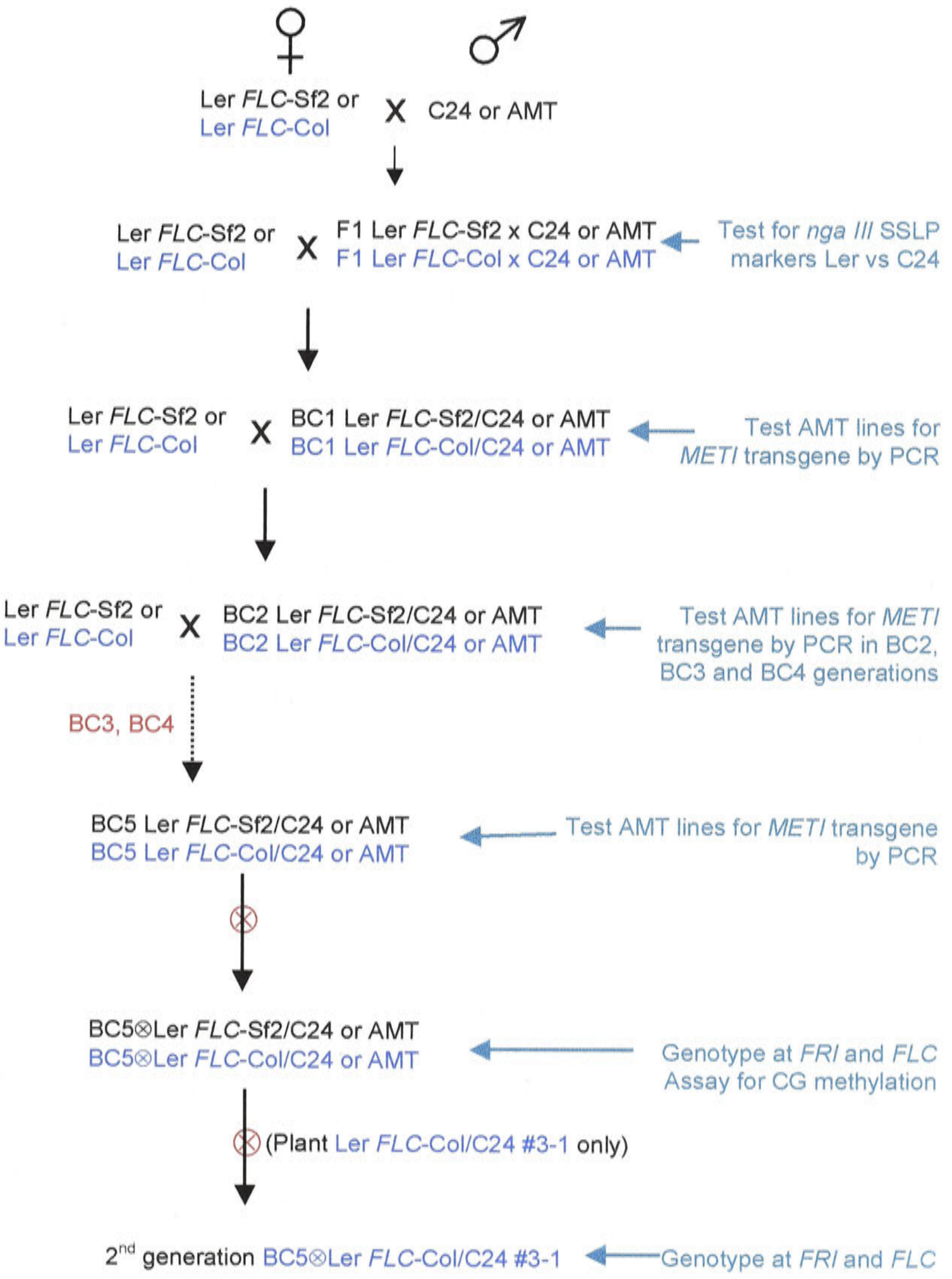
The near isogenic BC5 lines, differing by the presence of AMT or the *ddm1* mutation, were self-fertilised and a pooled sample of BC5⊗ progeny plants were analysed for their genotype at *FRI* and *FLC*. In each case the BC5 lines were genotyped to ensure that they were homozygous for both *FRI* and *FLC* from the Ler parental line, i.e. *FRI*-Ler and *FLC*-Col in the Ler *FLC*-Col backcrosses, and *FRI*-Ler and *FLC*-Sf2 in the Ler *FLC*-Sf2 backcrosses. This was done to ensure that results of the analysis, particularly flowering time experiments, were comparable with respect to the effects of *FRI* and *FLC*.

The backcrossing scheme and points at which screening was performed is outlined in Figure 4.10. A summary of all genotyped lines, method of determination and genotype at *FRI* and *FLC* is given in Table 4.3.

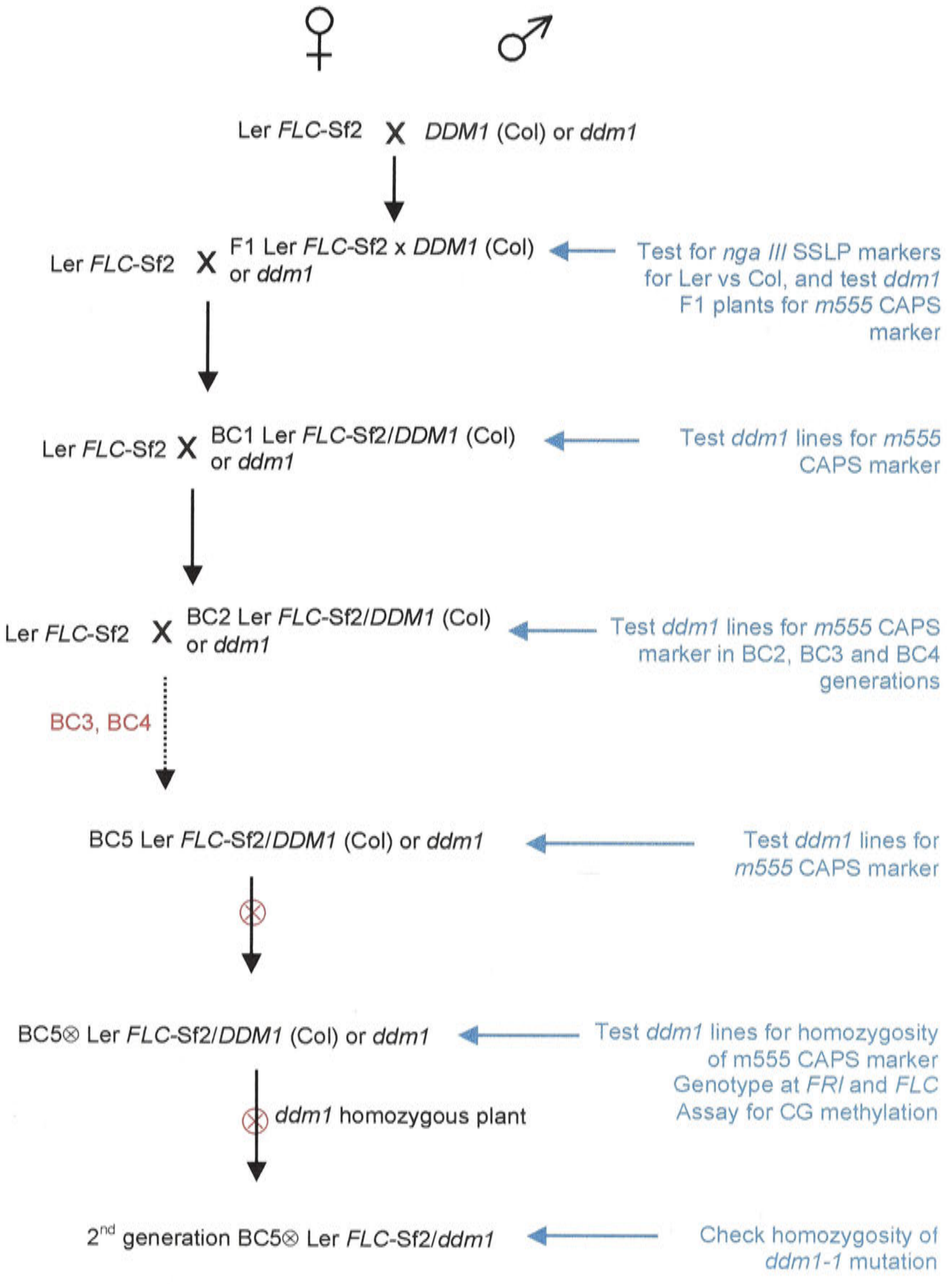
Figure 4.10 Diagrams outlining production of backcrossed lines, showing the timing and nature of screening procedures performed during the backcrossing process.

(A), Production of Ler *FLC*-Sf2/C24, Ler *FLC*-Sf2/AMT, Ler *FLC*-Col/C24 and Ler *FLC*-Col/AMT lines.

(B), Production of Ler *FLC*-Sf2/*DDM1* and Ler *FLC*-Sf2/*ddm1* lines.


♀
♂
Ler *FLC*-Sf2 or
Ler *FLC*-Col
X
C24 or AMT
Ler *FLC*-Sf2 or
Ler *FLC*-Col
X
F1 Ler *FLC*-Sf2 x C24 or AMT
F1 Ler *FLC*-Col x C24 or AMT
Test for *nga III* SSLP markers Ler vs C24
Ler *FLC*-Sf2 or
Ler *FLC*-Col
X
BC1 Ler *FLC*-Sf2/C24 or AMT
BC1 Ler *FLC*-Col/C24 or AMT
Test AMT lines for *METI* transgene by PCR
Ler *FLC*-Sf2 or
Ler *FLC*-Col
X
BC2 Ler *FLC*-Sf2/C24 or AMT
BC2 Ler *FLC*-Col/C24 or AMT
Test AMT lines for *METI* transgene by PCR in BC2, BC3 and BC4 generations
BC3, BC4
BC5 Ler *FLC*-Sf2/C24 or AMT
BC5 Ler *FLC*-Col/C24 or AMT
Test AMT lines for *METI* transgene by PCR
BC5⊗Ler *FLC*-Sf2/C24 or AMT
BC5⊗Ler *FLC*-Col/C24 or AMT
Genotype at *FRI* and *FLC*
Assay for CG methylation
⊗ (Plant Ler *FLC*-Col/C24 #3-1 only)
2nd generation BC5⊗Ler *FLC*-Col/C24 #3-1
Genotype at *FRI* and *FLC*

(A)


♀
♂
Ler *FLC*-Sf2 X *DDM1* (Col) or *ddm1*
Ler *FLC*-Sf2 X F1 Ler *FLC*-Sf2 x *DDM1* (Col) or *ddm1*
Test for *nga III* SSLP markers for Ler vs Col, and test *ddm1* F1 plants for *m555* CAPS marker
Ler *FLC*-Sf2 X BC1 Ler *FLC*-Sf2/*DDM1* (Col) or *ddm1*
Test *ddm1* lines for *m555* CAPS marker
Ler *FLC*-Sf2 X BC2 Ler *FLC*-Sf2/*DDM1* (Col) or *ddm1*
Test *ddm1* lines for *m555* CAPS marker in BC2, BC3 and BC4 generations
BC3, BC4
BC5 Ler *FLC*-Sf2/*DDM1* (Col) or *ddm1*
Test *ddm1* lines for *m555* CAPS marker
BC5⊗ Ler *FLC*-Sf2/*DDM1* (Col) or *ddm1*
Test *ddm1* lines for homozygosity of m555 CAPS marker
Genotype at *FRI* and *FLC*
Assay for CG methylation
ddm1 homozygous plant
2nd generation BC5⊗ Ler *FLC*-Sf2/*ddm1*
Check homozygosity of *ddm1-1* mutation

(B)

BC5⊗ Plant Line	*FRI* [a]	Method	*FLC* [a]	Method
Ler *FLC*-Col/C24 #3-1⊗[b]	Ler	Sequence polymorphism	Col	Sequence polymorphism
Ler *FLC*-Col/AMT #3	Ler	"	Col	"
Ler *FLC*-Sf2/C24 #1	Ler	"	Sf2	CAPS PCR
Ler *FLC*-Sf2/AMT #1	Ler	"	Sf2	"
Ler *FLC*-Sf2/*DDM1* #5	Ler	"	Sf2	SSLP analysis
Ler *FLC*-Sf2/*ddm1* #1-3⊗[b, c]	Ler	"	Sf2	"

Table 4.3 Genotyping of selfed BC5 lines used in final analysis.

[a] genotype at *FRI* or *FLC*; all lines were homozygous for the allele listed

[b] self-fertilised an extra generation

[c] this line was also homozygous for the *ddm1-1* mutation

4.3.3.1 Genotyping of Ler FLC-Col/AMT and Ler FLC-Col/C24

The BC5⊗ Ler *FLC*-Col/AMT and BC5⊗ Ler *FLC*-Col/C24 plants were genotyped to ensure they were homozygous for the *FLC*-Col allele and the *FRI* allele from the Ler *FLC*-Col parent. The *FLC* genotype was determined using PCR for sequence polymorphisms in a region of the *FLC* promoter which contains six polymorphisms between C24 and Col. Ler *FLC*-Col/AMT #3 was homozygous for the *FLC*-Col allele (Figure 4.11, Table 4.3). Sequencing of the *FLC* promoter PCR product amplified from pooled samples of two Ler *FLC*-Col/C24 lines, #1 and #3, consistently gave ambiguous sequence ("N") at two sites (Figure 4.11), suggesting that not all plants in this population were homozygous for *FLC*-Col. DNA extracted from six individual plants from the same generation of each line was subsequently sequenced with the *FLC* primers and one plant, #3-1, of the Ler *FLC*-Col/C24 #3 line was identified as homozygous for the *FLC*-Col allele at all six sites (Figure 4.11). Plant #3-1 was allowed to self fertilise and the resulting seed was used in the final experimental analyses.

The *FRI* genotype of the BC5⊗ Ler *FLC*-Col/AMT and BC5⊗ Ler *FLC*-Col/C24 plants was determined using PCR for a sequence polymorphism in the *FRI* allele. The *FRI* PCR product has a single base change from A (C24) to G (Ler) at position 1010 within the ERM/GRM motif of the *FRI* sequence (Genbank accession #AF228500). Ler *FLC*-Col/AMT #3 and the Ler *FLC*-Col/C24 selfed line #3-1 were homozygous for the *FRI*-Ler allele (Figure 4.12).

4.3.3.2 Genotyping of Ler FLC-Sf2/AMT and Ler FLC-Sf2/C24

The BC5⊗ Ler *FLC*-Sf2/AMT plants and the BC5⊗ Ler *FLC*-Sf2/C24 plants were genotyped to ensure they were homozygous for the *FLC* allele from Sf2 and the *FRI* allele

```
C24                    GAACTTGGGTTGATGTGAGGCACTATTAAGTAAAAAGCCATTG
Col                    GAACTTGGATTGATGTGGGGCACTATTAAGTAAAAAGCCACTG
Ler FLC-Col/AMT#3      GAACTTGGATTGATGTGGGGCACTATTAAGTAAAAAGCCACTG
Ler FLC-Col/C24#1      GAACTTGGNTTGATGTGGGGCACTATTAAGTAAAAAGCCACTG
Ler FLC-Col/C24#3      GAACTTGGNTTGATGTGGGGCACTATTAAGTAAAAAGCCACTG
Ler FLC-Col/C24#3-1    GAACTTGGATTGATGTGGGGCACTATTAAGTAAAAAGCCACTG

C24                    TACTACTTACATTTTAACTACATAATGTTAACTTATATAATATT
Col                    TACTACTTACATTTTAACTACAAAATGTTAACTTATATAATATT
Ler FLC-Col/AMT#3      TACTACTTACATTTTAACTACAAAATGTTAACTTATATAATATT
Ler FLC-Col/C24#1      TACTACTTACATTTTAACTACANAATGTTAACTTATATAATATT
Ler FLC-Col/C24#3      TACTACTTACATTTTAACTACANAATGTTAACTTATATAATATT
Ler FLC-Col/C24#3-1    TACTACTTACATTTTAACTACAAAATGTTAACTTATATAATATT

C24                    TATTGAATTCAGTATAGGGCACATGCCCTATCCATGACTAAC
Col                    TATTGAATTCAGTATAGGGCACATGCCCTACCCATGACTAAC
Ler FLC-Col/AMT#3      TATTGAATTCAGTATAGGGCACATGCCCTACCCATGACTAAC
Ler FLC-Col/C24#1      TATTGAATTCAGTATAGGGCACATGCCCTACCCATGACTAAC
Ler FLC-Col/C24#3      TATTGAATTCAGTATAGGGCACATGCCCTACCCATGACTAAC
Ler FLC-Col/C24#3-1    TATTGAATTCAGTATAGGGCACATGCCCTACCCATGACTAAC

C24                    GTGAGTCCG.CCCTGATAG
Col                    GTGAGTCCGCCCCTGATAG
Ler FLC-Col/AMT#3      GTGAGTCCGCCCCTGATAG
Ler FLC-Col/C24#1      GTGAGTCCGCCCCTGATAG
Ler FLC-Col/C24#3      GTGAGTCCGCCCCTGATAG
Ler FLC-Col/C24#3-1    GTGAGTCCGCCCCTGATAG
```

Figure 4.11 Sequence alignment of a section of the *FLC* promoter from backcrossed lines. Polymorphisms are shown in red (C24) or blue (Col). N (in orange) = sequence insufficiently clear.

C24	AATAAACCGGAGGGGGAACGTATGTGTGAGTTG
Col	AATAAACCGGAGGGGGAACGTATGTGTGAGTTG
AA sequence	N K P E G E R M C E L
Ler	AATAAACCGGAGGGGGGACGTATGTGTGAGTTG
AA sequence	N K P E G G R M C E L
Ler *FLC*-Col/AMT#3	AATAAACCGGAGGGGGGACGTATGTGTGAGTTG
Ler *FLC*-Col/C24#3-1	AATAAACCGGAGGGGGGACGTATGTGTGAGTTG
Ler *FLC*-Sf2/AMT#1	AATAAACCGGAGGGGGGACGTATGTGTGAGTTG
Ler *FLC*-Sf2/C24#1	AATAAACCGGAGGGGGGACGTATGTGTGAGTTG
Ler FLC-Sf2/ddm1#1	AATAAACCGGAGGGGGGACGTATGTGTGAGTTG
Ler *FLC*-Sf2/*DDM1*#5	AATAAACCGGAGGGGGGACGTATGTGTGAGTTG

Figure 4.12 Sequence alignment of a section of the *FRI* allele (Genbank accession #AF228500) and amino acid (AA) sequence from C24, Col, Ler and backcrossed lines. Within the ERM/GRM motif, C24 and Col have an A (in red), but the Ler allele has a G (shown in blue), leading to a substitution of a glycine for a glutamine.

from the Ler parent. The *FLC* genotype was determined by a CAPS PCR method, using primers that amplified a section of the *FLC* promoter. The C24 PCR product is cut twice by *Taa*I, producing fragments of 238, 49, and 25 bp. The Sf2 PCR product is cut three times, producing fragments of 157, 81, 49 and 25 bp. Ler *FLC*-Sf2/AMT #1 and #3 and Ler *FLC*-Sf2/C24 #1 and #2 were all homozygous for the Sf2 *FLC* allele (Figure 4.13). These four lines were then genotyped at the *FRI* allele. Lines Ler *FLC*-Sf2/AMT #1 and Ler *FLC*-Sf2/C24 #1 were homozygous for the Ler *FRI* allele (Figure 4.12) and were used in further analyses.

4.3.3.3 Genotyping of Ler FLC-Sf2/ddm1 and Ler FLC-Sf2/DDM1

The BC5⊗ Ler *FLC*-Sf2/*ddm1* and BC5⊗ Ler *FLC*-Sf2/*DDM1* plants were genotyped to ensure they had inherited the *FLC* allele from Sf2 and the *FRI* allele from the Ler parent. Despite using several primer combinations, no sequence polymorphisms could be detected between Col and Sf2 over ca. 2 kb of the *FLC* promoter region (results not shown). Instead, the inheritance of an SSLP marker, *nga 225*, closely linked to *FLC* on chromosome 5 (*Arabidopsis thaliana* Science Genome Map 9; TAIR, *www.arabidopsis.org*) was analysed in the BC5 selfed progeny. The lines were analysed to ensure that they had inherited *nga 225* (and therefore *FLC*) from the recurrent Ler parent, and not from Col. Lines Ler *FLC*-Sf2/*ddm1* #1 and #2 and Ler *FLC*-Sf2/*DDM1* #5 generated the same size band as Ler *FLC*-Sf2 (Figure 4.14, lanes 4, 6 and 7), indicating that they had inherited this chromosomal region from the recurrent Ler parent. The Ler *FLC*-Sf2/*DDM1* #12 line appeared to be heterozygous for the *nga 225* marker (Figure 4.14, lane 5) and was therefore excluded from further analysis.

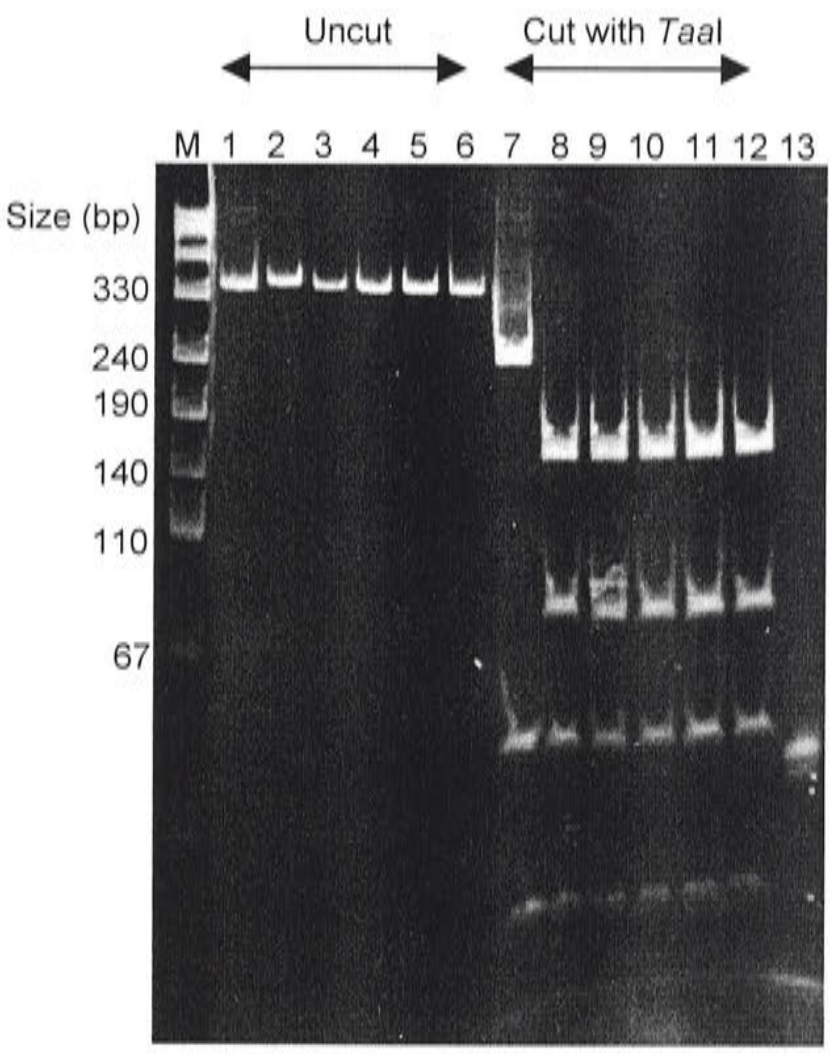


Figure 4.13 *FLC* CAPS analysis of BC5 selfed progeny plants on an 8% acrylamide gel. Lane M, marker pUC19/HpaII; lane 1 and 7, C24; lane 2 and 8, Ler *FLC*-Sf2; lanes 3-4 and 9-10, Ler *FLC*-Sf2/C24 #1 and #2; lanes 5-6 and 11-12, Ler *FLC*-Sf2/AMT #1 and #3; lane 13, water negative control containing primer-dimers. Lanes 1-6, uncut PCR product. Lanes 7-12, PCR product cut with *Taa*I. The C24 product is cut twice by *Taa*I but the Sf2 product is cut three times.

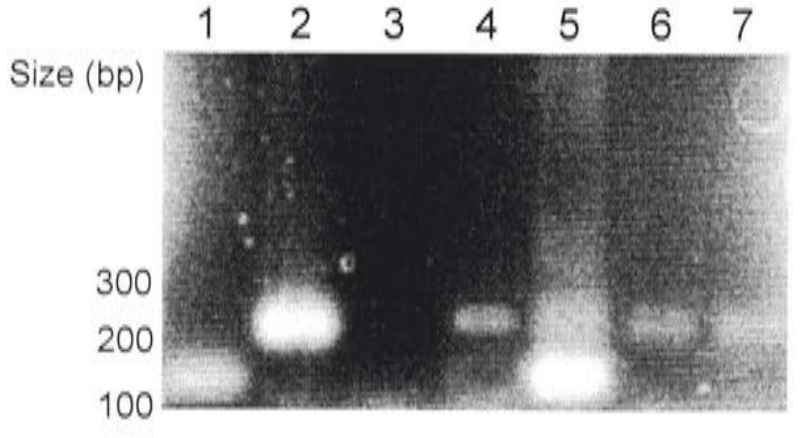


Figure 4.14 SSLP analysis of selfed BC5 lines using *nga 225* markers on a 2% agarose gel. Lane 1, Col; lane 2, Ler *FLC*-Sf2; lane 3, water negative control; lane 4, Ler *FLC*-Sf2/*DDM1* #5; lane 5, Ler *FLC*-Sf2/*DDM1* #12; lane 6, Ler *FLC*-Sf2/*ddm1* #1; lane 7, Ler *FLC*-Sf2/*ddm1* #2.

The *FRI* genotype of the *ddm1* and Col (*DDM1)* lines was determined using PCR for a sequence polymorphism in the *FRI* allele, which has a single base change from A (Col) to G (Ler) at the same position as the base change differentiating C24 and Ler. Lines Ler *FLC*-Sf2/*ddm1* #1 and Ler *FLC*-Sf2/*DDM1* #5 were homozygous for the *FRI* allele from Ler (Figure 4.12).

4.3.3.4 Generation of ddm1 homozygous lines

The backcrossed progeny of *ddm1* to Ler *FLC*-Sf2 were screened in each generation to ensure that the *DDM1* mutant allele (i.e. *ddm1-1*) had been inherited from the Col parent. As the *ddm1* mutation is recessive (Vongs *et al.,* 1993), the BC5 Ler *FLC*-Sf2/*ddm1* #1 line was allowed to self fertilise so that *ddm1* homozygous plants could be obtained. Individual progeny plants were screened using the *m555* CAPS PCR method to identify plants which were homozygous for the Col *m555* marker and would therefore carry the Col parent *ddm1* allele. Plant Ler *FLC*-Sf2/*ddm1* #1-3 was identified as homozygous at the Col *m555* locus, and therefore likely to be homozygous for the mutant *ddm1* allele (Figure 4.15). This plant was self-fertilised to provide second generation homozygous seed for final analysis.

The progeny of line #1-3 were then tested by PCR for the sequence polymorphism of the *ddm1-1* mutation to confirm that the line was homozygous for the *ddm1-1* mutant allele. The *ddm1-1* allele results from a G→A transition at position 3637 of the Genbank accession #AF143940 (E Richards, personal communication), resulting in a substitution of a tyrosine for a cysteine. DNA samples from the original *ddm1* line, wild type Col and the BC5⊗ line were sequenced using primers flanking the *ddm1-1* mutation. The BC5⊗Ler *FLC*-Sf2/*ddm1* #1-3 line was confirmed as homozygous for the *ddm1-1* allele (Figure 4.16).

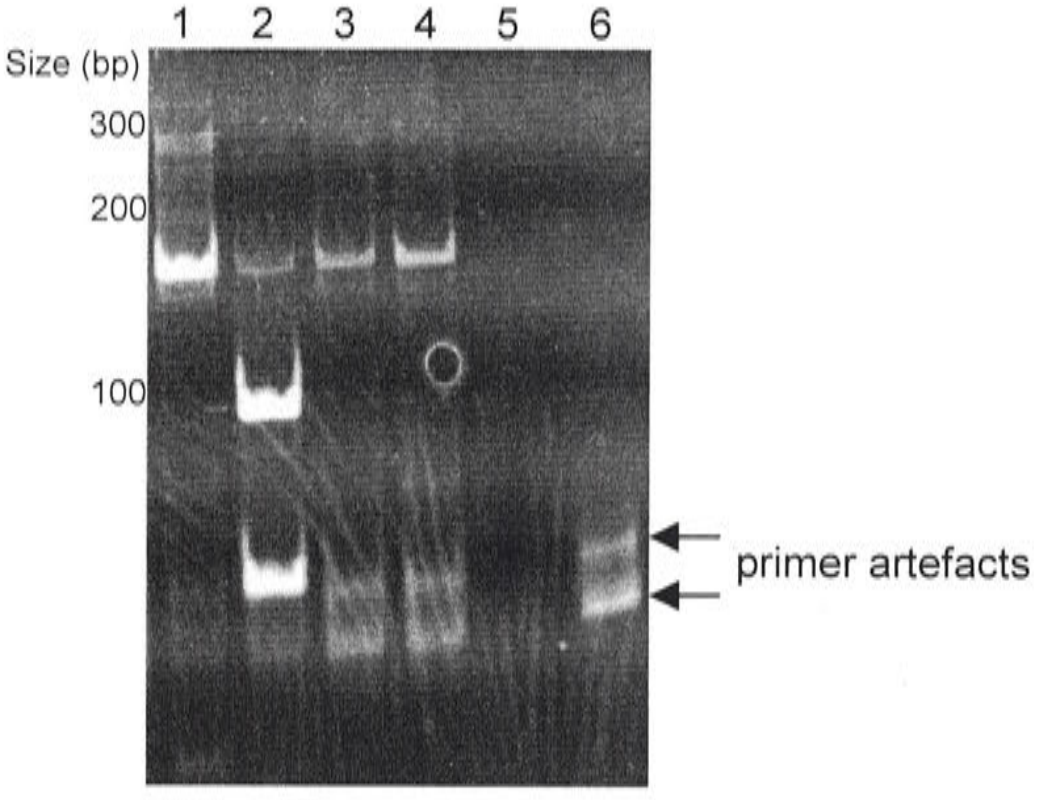


Figure 4.15 *m555* CAPS analysis of selfed BC5 Ler *FLC*-Sf2/*ddm1* #1 progeny plant #3 on an 8% acrylamide gel. Lane 1, Col; lane 2, Ler; lane 3, *ddm1* #10 progenitor line; lane 4, BC5 Ler *FLC*-Sf2/*ddm1* #1 progeny plant #3; lane 5, empty; lane 6, water negative control (lane 6 contains primer artefacts, black arrows).

Note: the digest of Ler is incomplete, hence a non-stoichiometric band is seen at 150 bp in lane 2.

Col	AATCTTACTGCTGCTGATACATGCATCCTCTATGACAGC
AA sequence	N L T A A D T C I L Y D S
ddm1 #10	AATCTTACTGCTGCTGATACATACATCCTCTATGACAGC
AA sequence	N L T A A D T Y I L Y D S
Ler FLC-Sf2/ddm1#1-3	AATCTTACTGCTGCTGATACATACATCCTCTATGACAGC
AA sequence	N L T A A D T Y I L Y D S

Figure 4.16 Sequence alignment of a fragment of the *DDM1* locus in Col, *ddm1* and BC5 Ler *FLC*-Sf2/*ddm1* #1-3 showing the site of the *ddm1-1* mutation. The mutation site for Col is shown in blue, and for *ddm1* in red. The *ddm1-1* mutation, which occurs at nucleotide 3637 of the Genbank accession #AF143940, results in substitution of an A for a G, leading to a substitution of a tyrosine for a cysteine.

4.3.4 Methylation levels are reduced in backcrossed lines containing AMT or *ddm1*

Both AMT and *ddm1* are reported to reduce methylation within repetitive sequences (Finnegan *et al.*, 1996; Vongs *et al.*, 1993). DNA from each of the backcrossed lines was analysed for levels of methylation within repetitive DNA sequences at CG sites using the methylation-sensitive enzymes *Msp*I and *Hpa*II and by hybridisation to a 180 bp centromeric repeat probe (Martinez-Zapater et al., 1986). *Msp*I cuts the sequence CCGG whether or not the internal cytosine is methylated, but *Hpa*II only cuts unmethylated CCGG sites. Figure 4.17 shows the methylation levels of the control lines, Ler *FLC*-Col/C24, Ler *FLC*-Sf2/C24 and Ler *FLC*-Sf2/*DDM1*. It is clear from this figure that none of the control lines have a decrease in CG methylation, as the centromeric repeat is not cleaved by *Hpa*II. The Ler *FLC*-Col/AMT, Ler *FLC*-Sf2/AMT and Ler *FLC*-Sf2/*ddm1* lines were assayed in the same manner. All lines show evidence of reduced methylation at CG sites (Figure 4.18). However, in the Ler *FLC*-Col/AMT and the *Ler FLC*-Sf2/*ddm1* lines the extent of demethylation is much greater than in the Ler *FLC*-Sf2/AMT line (Figure 4.18, lanes 3 and 4). In this line, the majority of the centromeric repeat DNA remained uncut by *Hpa*II, as judged by the intense hybridisation at the region of the gel corresponding to uncut DNA, and the relatively weak hybridisation to the ladder of bands. In contrast, in the other lines the intensity of hybridisation to *Msp*I and *Hpa*II cut DNA was comparable.

The reason for the lack of demethylation in the Ler *FLC*-Sf2/AMT line has not been determined, but it is possible that the AMT transgene has lost efficacy in this line. In the parental line, T3#10.5 (Finnegan *et al.*, 1996), which has three copies of T-DNA, the selectable marker gene *NptII* has become hypermethylated and silenced (EJ Finnegan, personal communication). Perhaps during the backcrossing of the Ler *FLC*-Sf2/AMT line,

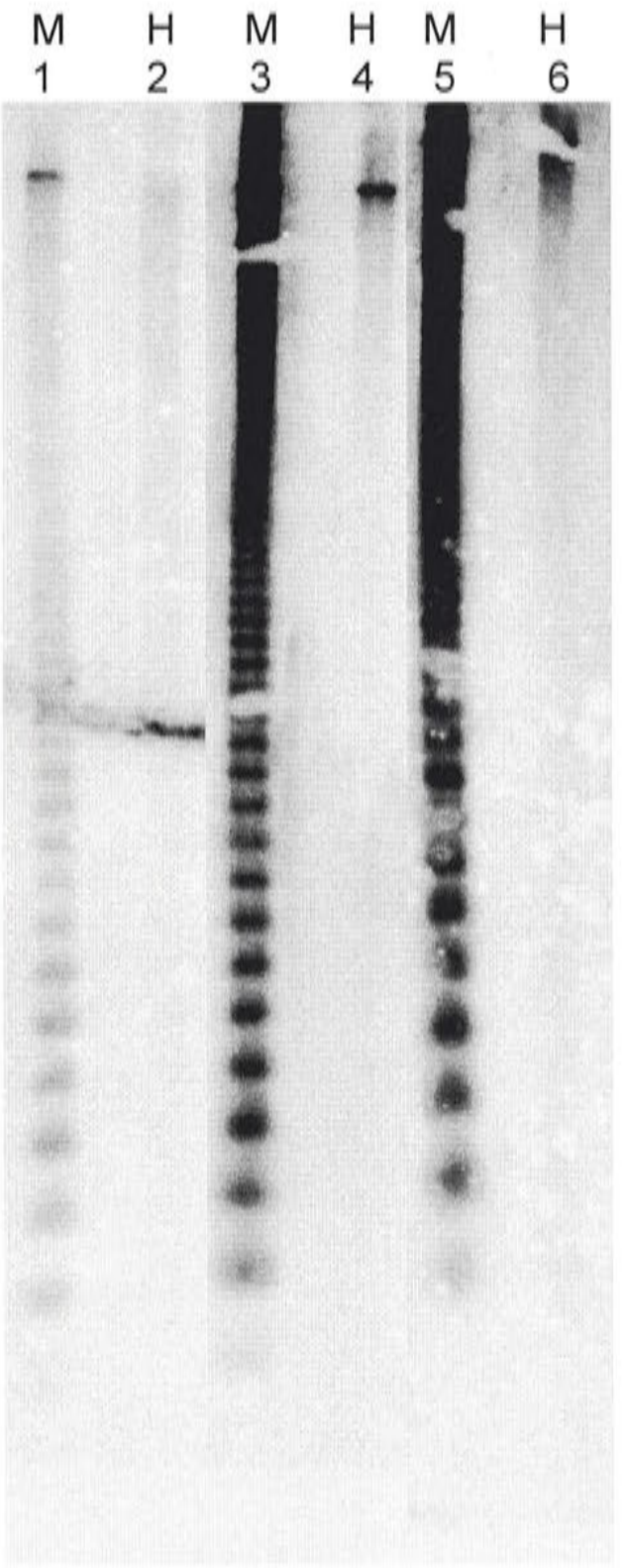


Figure 4.17 Analysis of methylation at CG sites in DNA from BC5 selfed lines by *Msp*I (M) and *Hpa*II (H) enzyme digestion and hybridisation to a 180 bp ribosomal repeat probe. Lane 1 & 2, Ler *FLC*-Col/C24; lane 3 & 4, Ler *FLC*-Sf2/C24; lane 5 & 6, Ler *FLC*-Sf2/*DDM1*.

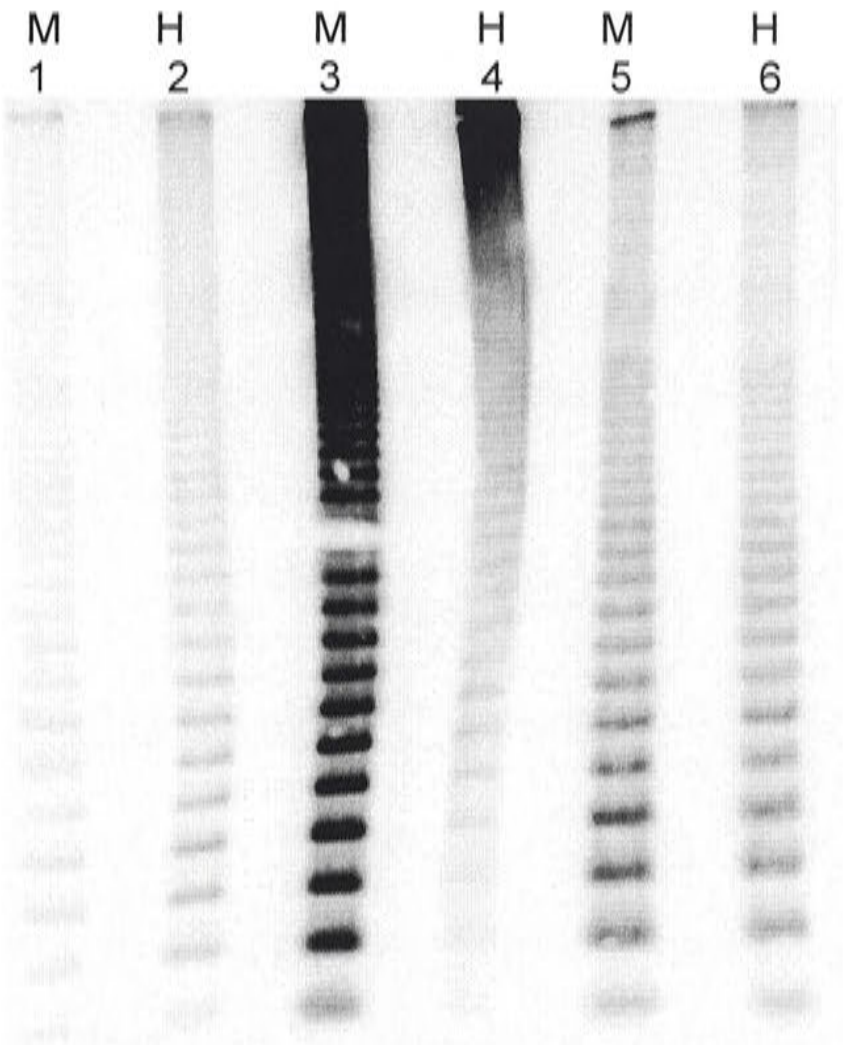


Figure 4.18 Analysis of methylation at CG sites in DNA from BC5 selfed lines by *Msp*I (M) and *Hpa*II (H) enzyme digestion of DNA and hybridisation to a 180 bp ribosomal repeat probe. Lanes 1 & 2, Ler *FLC*-Col/AMT; lanes 3 & 4, Ler *FLC*-Sf2/AMT; lanes 5 & 6, Ler *FLC*-Sf2/*ddm1*.

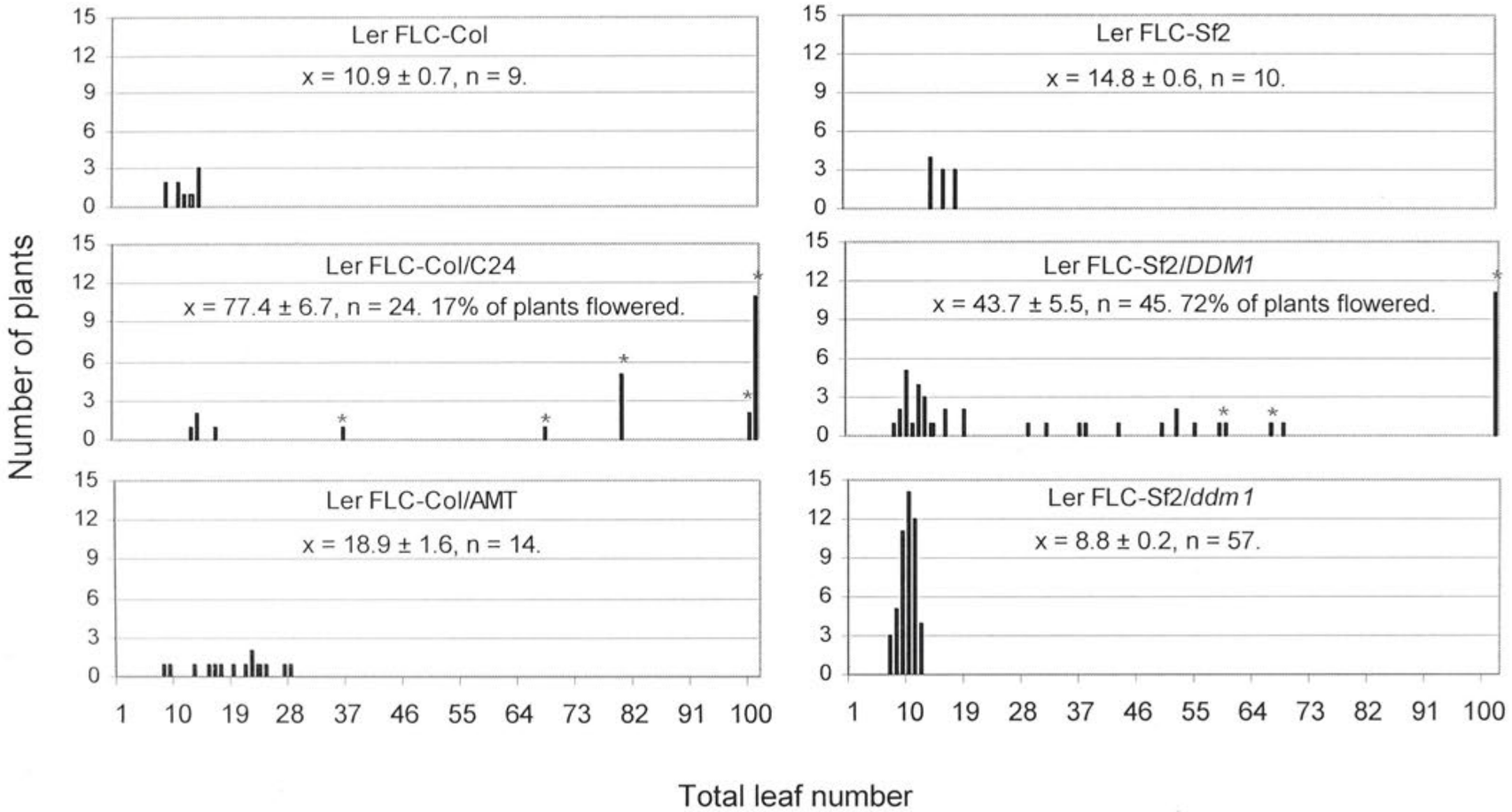
the AMT transgene has also become hypermethylated and silenced. Regardless of the reason for the lack of demethylation in this line, it was excluded from further analysis. The remainder of the investigation focussed on comparing the Ler *FLC*-Col/AMT and the *Ler FLC*-Sf2/*ddm1* lines, which had comparable levels of demethylation at CG sites within repetitive centromeric sequences. Although these lines differ in the original source of the *FLC* allele, both the Col and Sf2 *FLC* alleles delay flowering in the Ler background (Koornneef *et al.*, 1994; Lee *et al.*, 1994) and are expressed at similar levels (Sheldon *et al.*, 1999), so are therefore comparable.

4.3.5 Flowering is promoted in AMT and *ddm1* mutant backgrounds

To analyse the effect of the AMT transgene and the *ddm1* mutation on flowering, the flowering time of the Ler *FLC*-Col and Ler *FLC*-Sf2 parental lines was compared to that of the appropriate backcrossed lines in LD conditions. Ler *FLC*-Col flowered with 10.9 leaves, slightly earlier than Ler *FLC*-Sf2, which flowered with 14.8 leaves (Figure 4.19). Backcrossing C24 or Col to the parental lines delayed flowering on average compared to the parental lines, to the extent that 83 % of the Ler *FLC*-Col/C24 plants and 28 % of the Ler *FLC*-Sf2/*DDM1* plants did not flower within the time of the experiment (Figure 4.19; plants marked with an asterisk). The leaf number of these lines is therefore an underestimation.

There was a spread of flowering, such that some plants of the Ler *FLC*-Sf2/*DDM1* line flowered earlier than or at the same time as the parental line. A similar spread was seen in the Ler *FLC*-Col/C24 line, except that no plants flowered earlier than the parental line. Figures 4.20 and 4.21 show some of the variation in flowering time observed in these lines. When C24 and Ler are crossed, genes other than *FLC* and *FRI* affect flowering time

Figure 4.19 Flowering time expressed as total number of leaves of parental lines and BC5 selfed plants, grown in LD conditions. Plants marked * did not flower within the time of the experiment and were beginning to senesce when the experiment was terminated. Plants which had a total leaf number of greater than 100 are grouped together. Means +/- S.E. are given below each genotype. When calculating the means for Ler *FLC*-Col/C24 and Ler *FLC*-Sf2/*DDM1*, leaf numbers from plants that did not bolt within the time of the experiment were also included.


Ler FLC-Col
x = 10.9 ± 0.7, n = 9.
Ler FLC-Sf2
x = 14.8 ± 0.6, n = 10.
Ler FLC-Col/C24
x = 77.4 ± 6.7, n = 24. 17% of plants flowered.
Ler FLC-Sf2/DDM1
x = 43.7 ± 5.5, n = 45. 72% of plants flowered.
Ler FLC-Col/AMT
x = 18.9 ± 1.6, n = 14.
Ler FLC-Sf2/ddm1
x = 8.8 ± 0.2, n = 57.
Number of plants
Total leaf number

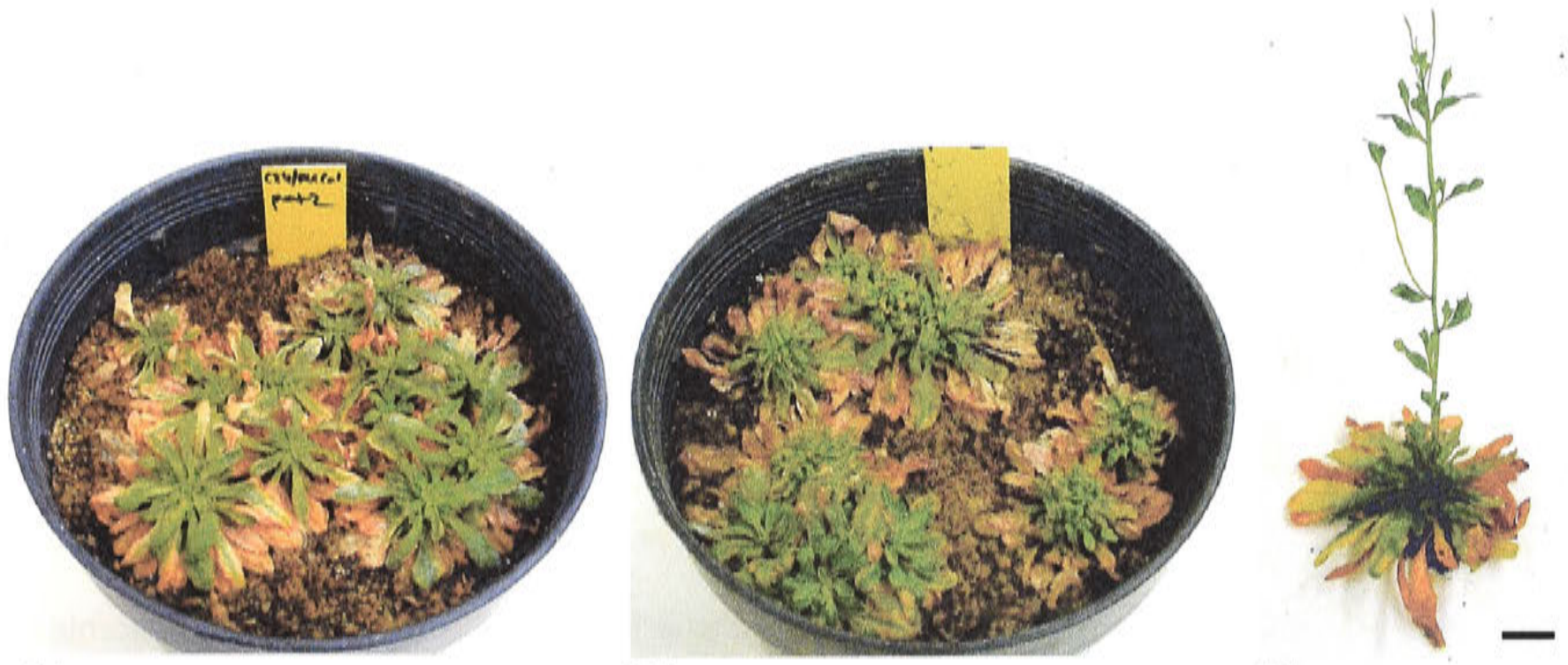
(A) (B)

(C) (D)

Figure 4.20 Five week old BC5 selfed plants grown in 6-inch diameter pots.

(A), Ler *FLC*-Col/C24. **(B)**, Ler *FLC*-Col/AMT. **(C)**, Ler *FLC*-Sf2/*DDM1*.

(D), Ler *FLC*-Sf2/*ddm1*.



(A) (B) (C)

Figure 4.21 Four month old BC5 selfed plants grown in 6-inch diameter pots.

(A), Ler *FLC*-Col/C24. **(B)**, Ler *FLC*-Sf2/DDM1. **(C)**, Individual Ler *FLC*-Sf2/DDM1 plant with >100 leaves. Scale bar in (C) = 1 cm.

(EJ Finnegan, personal communication); presumably, this also occurs in Col x Ler crosses. The Ler *FLC*-Col/C24 line appears to be segregating for a dominant late flowering gene, as most of the plants are late flowering, whereas the Ler *FLC*-Sf2/*DDM1* line appears to be segregating for a recessive late flowering gene, as most of the plants are early flowering (Figure 4.19).The observed delay in flowering time of the control BC lines was unexpected, as after five backcrosses only 1.5 % ($1/64^{th}$) of the C24 or Col genomes should still be present. These data suggest that control plants and those with low methylation levels may not be isogenic for all genes that affect flowering time.

The backcrossed lines with low methylation levels flowered significantly earlier than the Ler *FLC*-Col/C24 and Ler *FLC*-Sf2/*DDM1* lines (Figures 4.19 and 4.20), indicating that demethylation promoted flowering. In the Ler *FLC*-Col/AMT line, flowering was promoted almost to that of the parental line, whereas in the Ler *FLC*-Sf2/*ddm1* line, flowering was promoted to an even greater extent than the parental line (Figure 4.19). These results show that both the AMT construct and the *ddm1* mutation can cause early flowering in a genetic background that has equivalent *FRI* and *FLC* alleles. This will be discussed further in section 4.3.7. As the Ler *FLC*-Col parental line was earlier flowering than the Ler *FLC*-Sf2 line, it might have been expected that the *FLC*-Col/AMT line would flower earlier than the Ler *FLC*-Sf2/*ddm1* line, but the opposite occurred; the Ler *FLC*-Sf2/*ddm1* line flowered with 8.8 leaves, compared to 18.9 leaves of the Ler *FLC*-Col/AMT line (Figure 4.19). This observation will be considered in section 4.3.10.

4.3.6 Leaf morphology is affected in AMT and *ddm1* mutant backgrounds

An effect on flowering time was not the only phenotype observed in the Ler *FLC*-Col/AMT and Ler *FLC*-Sf2/*ddm1* lines. Backcrossing C24 or Columbia to parental lines had little

effect on leaf shape, but backcrossing *AMT* or *ddm1* to the parental lines resulted in altered leaf dimensions (Figure 4.22). Leaves of the AMT and *ddm1* BC5 plants were both narrower and shorter than the leaves of the controls, resulting in a smaller, rounded leaf shape. Such phenotypes have previously been observed in both *ddm1* mutants (Kakutani *et al.*, 1996) and AMT plants (Finnegan *et al.*, 1996) with low methylation levels. Therefore, it seems likely that both the leaf shape phenotype and the promotion of flowering observed in the AMT and *ddm1* BC5 lines were due to demethylation, and did not result from an unrelated effect of the backcrossing regime.

4.3.7 Flowering time is correlated with down-regulation of *FLC* expression in AMT and *ddm1* mutant backgrounds

As both the AMT and *ddm1* BC5⊗ lines flowered earlier than the control lines, a molecular explanation was sought to explain this behaviour. The level of *FLC* expression is correlated with flowering time and is down-regulated by demethylation in C24 (Sheldon *et al.*, 1999); therefore, the *FLC* expression profile of the different backcrossed lines was analysed.

In the Ler *FLC*-Col/C24 and Ler *FLC*-Sf2/*DDM1* control lines, *FLC* was highly expressed (Figure 4.23). The expression of *FLC* in the Ler *FLC*-Col/C24 line appeared to be almost double that of the Ler *FLC*-Sf2/*DDM1* line (Figure 4.23, lane 1 vs 3). The relative *FLC* expression levels of the two control lines correlate well with the flowering time results (Figure 4.19); only 17 % of Ler *FLC*-Col/C24 plants flowered during the time of the experiment, compared to 72 % of the Ler *FLC*-Sf2/*DDM1* plants. Even though more RNA was loaded in the Ler *FLC*-Col/C24 sample (Figure 4.23, lane 1 vs 3), this would account for only some of this difference. The difference also relates to the origins of *FLC* and is

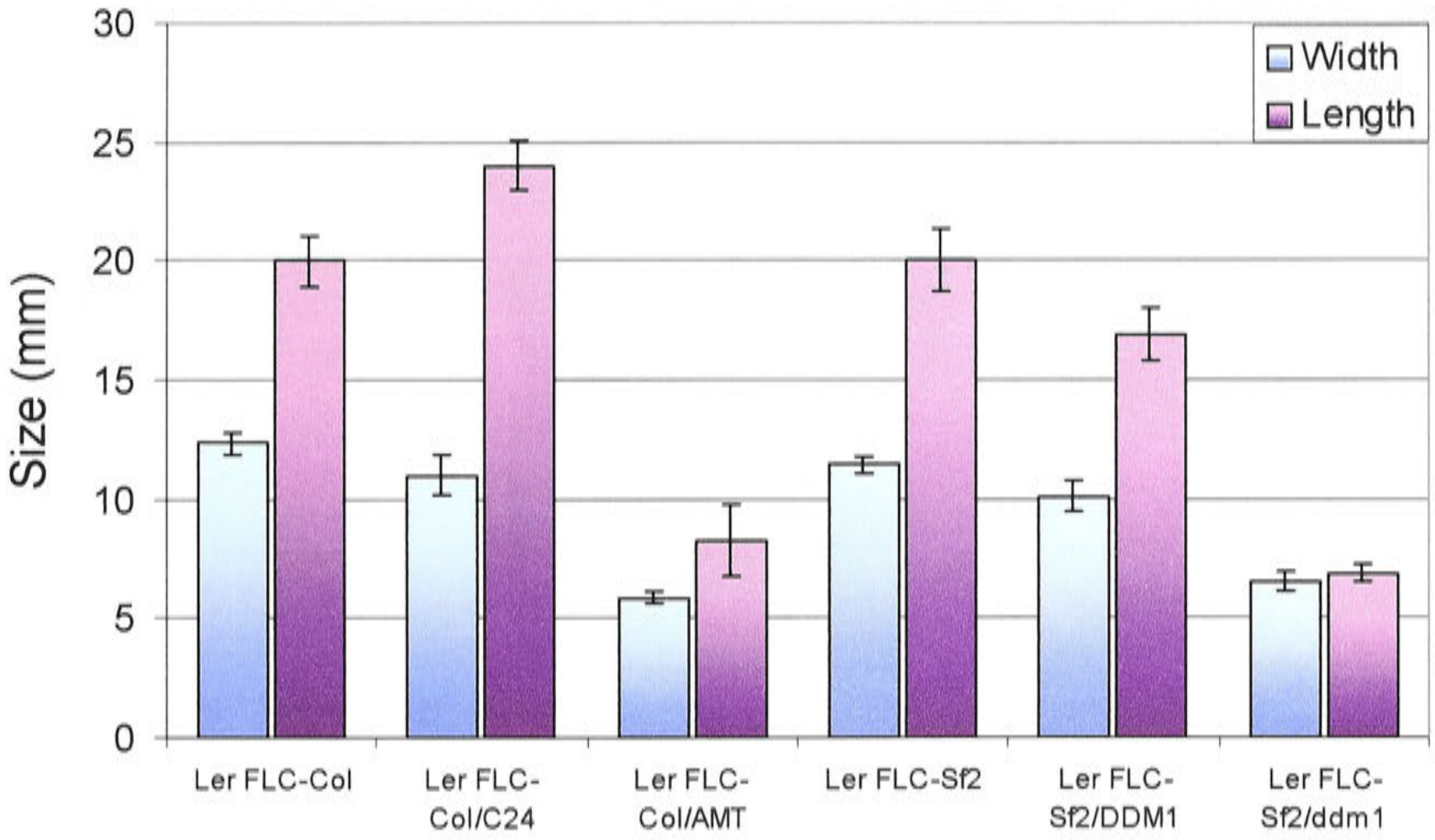


Figure 4.22 Leaf dimensions (length and width) of parental lines and BC5 selfed plants. Measurements were taken of the three youngest rosette leaves of three plants per line, hence bars represent the average of nine leaves per line. Measurements were taken after bolting had occurred. Width was measured at the centre of the leaf and length was measured as the distance from the base of the petiole to the tip of the leaf.

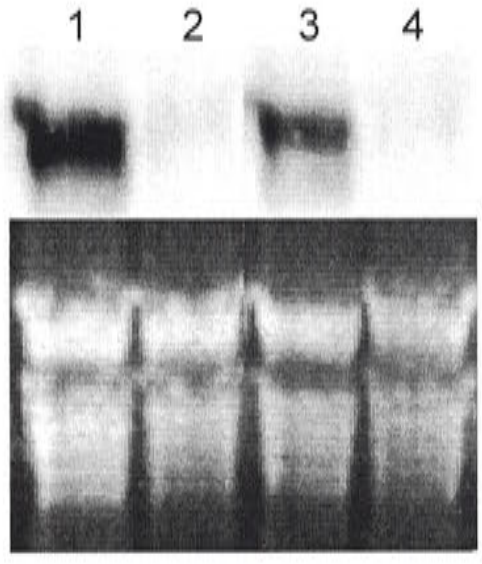


Figure 4.23 Expression of *FLC* in three-week old seedlings of BC5 selfed lines.

RNA was separated on a 1.1 % formaldehyde gel and stained with EtBr. RNA was then transferred to a filter and hybridized to the *FLC* riboprobe. Lane 1, Ler *FLC*-Col/C24; lane 2, Ler *FLC*-Col/AMT; lane 3, Ler *FLC*-Sf2/*DDM1*; lane 4, Ler *FLC*-Sf2/*ddm1*.

consistent with the level of *FLC* expression observed in the Ler *FLC*-Col parental line, which is higher than that of the Ler *FLC*-Sf2 parental line (Sheldon *et al.*, 1999). Despite this, Ler *FLC*-Col flowered earlier than Ler *FLC*-Sf2 (Figure 4.19); perhaps this indicates the presence of modifying gene/s in the *FLC*-Col line that influence flowering time and overcome the effect of high *FLC* expression.

The level of *FLC* expression was next analysed in the backcrossed lines with low methylation levels. *FLC* expression was greatly reduced in the Ler *FLC*-Col/AMT line compared to the Ler *FLC*-Col/C24 line (Figure 4.23, lanes 1 and 2). This is consistent with AMT causing a reduction in *FLC* expression (Sheldon *et al.*, 1999). The reduction in *FLC* expression in this line compared to the Ler *FLC*-Col/C24 control line correlates with the promotion of flowering observed. However, despite the low levels of *FLC* expression in the Ler *FLC*-Col/AMT plants, they did not flower as early, on average, as the parental Ler *FLC*-Col line, even though this line is reported as having a moderate level of *FLC* expression (Sheldon *et al.*, 1999). See section 4.3.10 for further discussion of this observation.

The expression of *FLC* was next analysed in the Ler *FLC*-Sf2/*ddm1* line. As for the Ler *FLC*-Col/AMT line, the expression of *FLC* was greatly reduced, to an almost undetectable level, as a consequence of the *ddm1* mutation (Figure 4.23, lanes 3 and 4). This shows that the *ddm1* mutation can result in the down-regulation of *FLC* expression. The decrease in *FLC* expression in the Ler *FLC*-Sf2/*ddm1* line correlated well with the promotion of flowering. In this line, the absence of detectable *FLC* expression resulted in very early flowering, with all of the Ler *FLC*-Sf2/*ddm1* plants flowering earlier than the earliest Ler *FLC*-Sf2 parental plant (Figure 4.19).

4.3.8 *UFC* expression is also down-regulated in AMT and *ddm1* mutant backgrounds

It has not yet been determined whether the reduction in *FLC* expression as a result of demethylation involves demethylation of an upstream repressor, or occurs via an interaction between DNA methylation and the chromatin structure surrounding *FLC*. To begin to understand how *FLC* expression might decrease in response to demethylation, expression analysis of *UFC* was carried out on the backcrossed lines. *UFC* is the gene immediately upstream of *FLC* and like *FLC*, is also down-regulated in C24 AMT plants (EJ Finnegan, personal communication), *UFC* is expressed at a high level in both the Ler *FLC*-Col/C24 and Ler *FLC*-Sf2/*DDM1* lines (Figure 4.24a, lanes 1 and 3). Like *FLC*, expression of *UFC* is reduced in both the Ler *FLC*-Col/AMT and Ler *FLC*-Sf2/*ddm1* lines (Figure 4.24a, lanes 2 and 4) compared to the controls. As *UFC* is adjacent to *FLC*, these data suggest that demethylation, whether caused by AMT or *ddm1*, is likely to have an effect over the *UFC-FLC* region, rather than affecting *FLC* specifically.

4.3.9 *SOC1* expression is up-regulated in AMT and *ddm1* mutant backgrounds

To determine whether the down-regulation of *FLC* in the Ler *FLC*-Col/AMT and Ler *FLC*-Sf2/*ddm1* lines was associated with up-regulation of *SOC1*, a promoter of flowering that is repressed by *FLC* (Onouchi *et al.*, 2000; Samach *et al.*, 2000), the expression of *SOC1* was investigated. The level of *SOC1* expression increased in both the Ler *FLC*-Col/AMT and Ler *FLC*-Sf2/*ddm1* lines compared to the control lines (Figure 4.24b). The increase in *SOC1* expression correlates with both the reduction in *FLC* expression and the promotion of flowering in the two backcrossed lines compared to the control lines. This result indicates that regardless of whether *FLC* expression is down-regulated by AMT or *ddm1*-

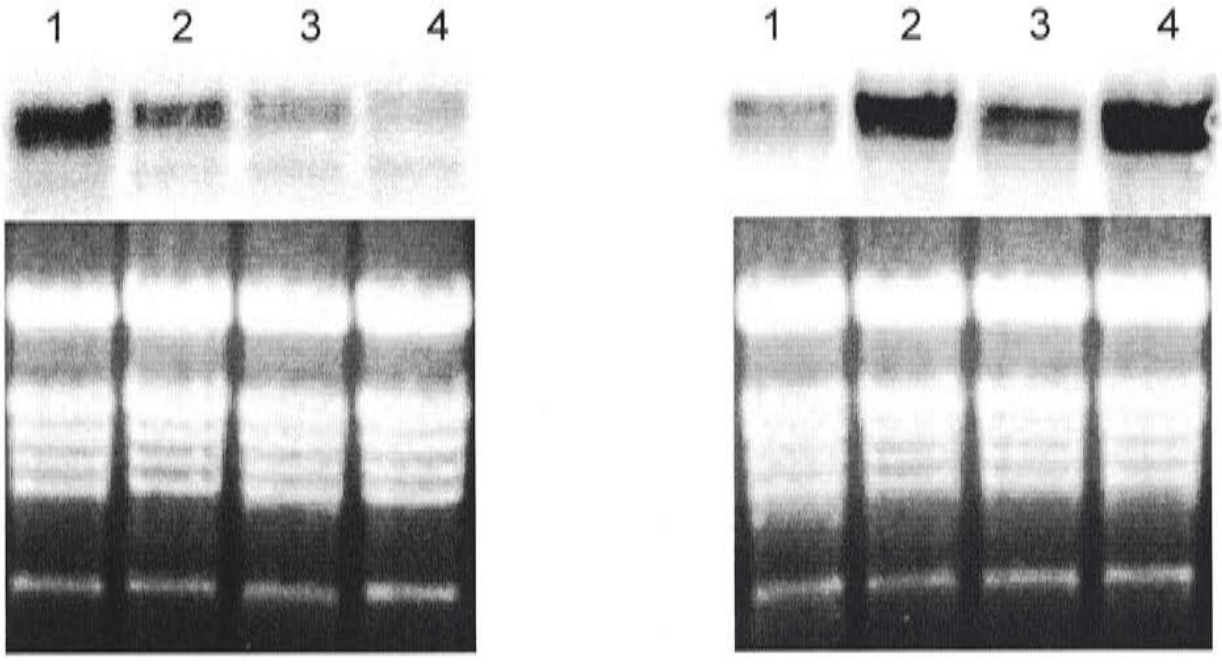


Figure 4.24 Expression of *UFC* and *SOC1* in three-week old seedlings of BC5 selfed lines. RNA was separated on a 1.1 % formaldehyde gel and stained with EtBr. RNA was transferred to a filter and hybridized to **(A)** *UFC* or **(B)** *SOC1* riboprobes.
Lane 1, Ler *FLC*-Col/C24; lane 2, Ler *FLC*-Col/AMT; lane 3, Ler *FLC*-Sf2/*DDM1*; lane 4, Ler *FLC*-Sf2/*ddm1*.

induced demethylation, as predicted, the immediate downstream effect is the same; that is, *SOC1* expression increases as expression of *FLC*, a repressor of *SOC1*, increases.

4.3.10 *FWA* is demethylated and expressed only in an AMT background

As described earlier, the AMT transgene causes demethylation of both single-copy and repeated sequences (Finnegan *et al.*, 1996; Ronemus *et al.*, 1996) but the *ddm1* mutation in early generations results only in the demethylation of repeated sequences (Vongs *et al.*, 1993). Multiple copy sequences were demethylated in both the AMT and *ddm1* backcrossed lines relative to the controls (section 4.3.4). The methylation status of a single-copy sequence, *FWA*, was next analysed in the AMT line and in the *ddm1* backcrossed line, where *ddm1* had been homozygous for only two generations. The methylation status was analysed by *Msp*I and *Hpa*II digestion and hybridisation to an *FWA* probe. In the Ler *FLC*-Sf2/*ddm1* line, *FWA* is digested by *Msp*I, but not by *HpaII,* and is therefore methylated (Figure 4.25, lanes 3 and 4). This is consistent with the *ddm1* mutation only affecting multiple-copy sequences in early generations (Vongs *et al.*, 1993). In contrast, in the Ler *FLC*-Col/AMT line, *FWA* is digested by both *Msp*I and *HpaII*, and is therefore demethylated (Figure 4.25, lanes 1 and 2). This is consistent with AMT causing demethylation of single-copy sequences and repeated sequences equally (Finnegan et al., 1996; Ronemus *et al.*, 1996).

In conjunction with analysis of the methylation status of *FWA*, the expression of *FWA* was analysed using RT-PCR. *FWA* was not expressed in either of the control lines (Figure 4.26, lanes 2 and 4). This is consistent with earlier reports showing that *FWA* is not normally expressed in the Ler background (Soppe *et al.*, 2000). In the Ler *FLC*-Sf2/*ddm1* line, *FWA* was not expressed (Figure 4.26, lane 5), correlating with the methylated state of

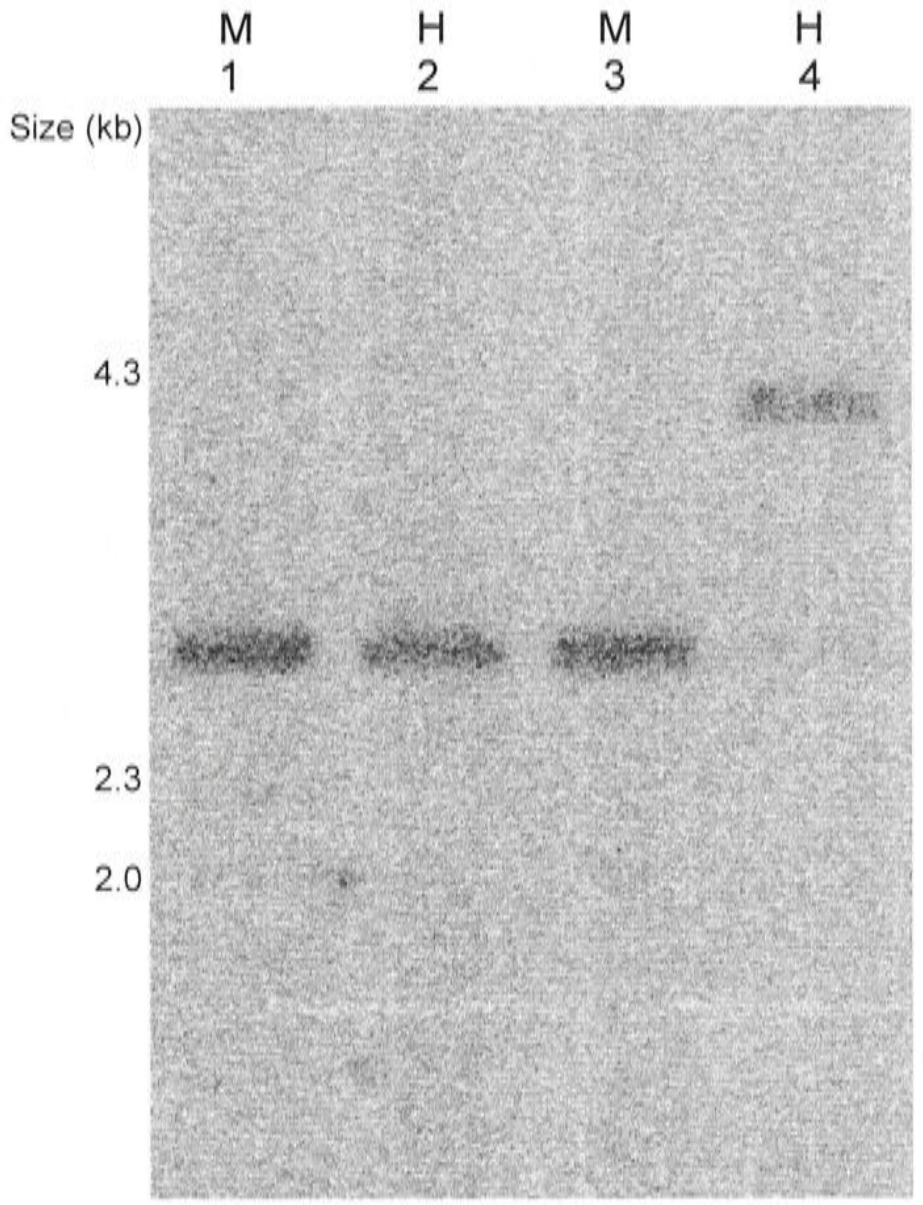


Figure 4.25 Analysis of CG methylation status of part of the coding region of the *FWA* gene by *Msp*I (M) and *Hpa*II (H) digestion and hybridisation to the VE030 *FWA* probe.

Lane 1 and 2, Ler *FLC*-Col/AMT; lane 3 and 4, Ler *FLC*-Sf2/*ddm1*.

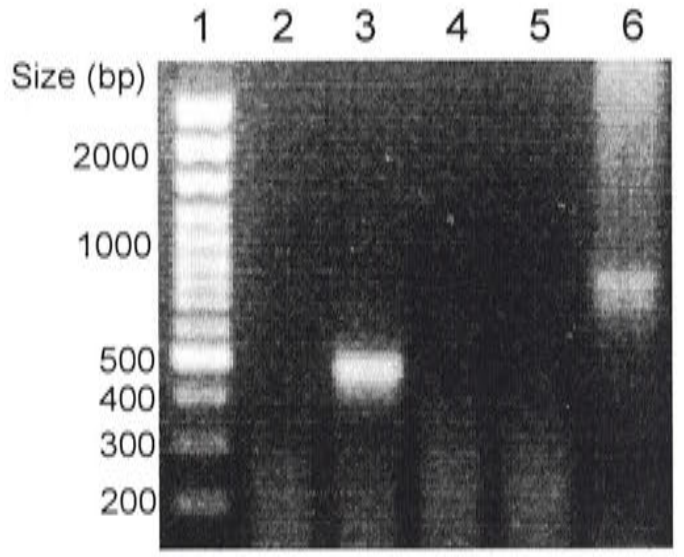


Figure 4.26 RT-PCR amplification of *FWA* in BC5 lines. Lane 1, 'Gene Ruler' 100 bp DNA ladder; lane 2, Ler *FLC*-Col/C24; lane 3, Ler *FLC*-Col/AMT; lane 4, Ler *FLC*-Sf2/*DDM1*; lane 5, Ler *FLC*-Sf2/*ddm1*; lane 6, genomic DNA control.

the *FWA* gene (Figure 4.25). However, *FWA* is expressed in the Ler *FLC*-Col/AMT line (Figure 4.26, lane 3), correlating with the demethylated state of the *FWA* gene (Figure 4.25). The expression of *FWA* in the Ler *FLC*-Col/AMT line provides an explanation for why this line flowered later than the Ler *FLC*-Col parental line, despite the AMT line having lower levels of *FLC* expression than the parental line (not shown; refer to Sheldon *et al.*, 1999). The expression of *FWA* in the Ler *FLC*-Col/AMT line also correlates well with the delayed flowering of this line compared to the Ler *FLC*-Sf2/*ddm1* line (Figure 4.19), considering that they have comparable levels of *FLC* expression (Figure 4.23, lanes 2 and 4).

4.4 Discussion

Demethylation in the C24 and Columbia ecotypes of *Arabidopsis* has opposing effects on flowering time. In C24, a loss of methylation resulting from introduction of a *METI* antisense (AMT) construct causes early flowering (Finnegan *et al.*, 1998), whereas introduction of a similar AMT construct delays flowering in Col (this chapter; Ronemus *et al.*, 1996). Delayed flowering is also observed in the *ddm1* mutant, but only after several generations of inbreeding (Kakutani, 1997). The results presented here show that this is a combination of both the genetic background of each ecotype and the mechanisms causing demethylation. The production of near-isogenic lines has allowed a direct comparison of the effect of demethylation caused by either an AMT construct or a mutation in the chromatin-remodelling enzyme *DDM1*. Although the genetic backgrounds of the near isogenic lines are similar, they are not exactly the same. This is due not only to the different origins of *FLC*, but also because it is unknown how much of chromosome V of the Ler *FLC*-Col parental line is contributed by Col. However, the five backcrosses to the parental Ler lines should have removed 98.5 % of the C24 and Col genomes introduced in

the F1 progeny, making the genetic backgrounds of the backcrossed lines effectively comparable.

Backcrossing AMT and *ddm1* to Ler *FLC*-Col and Ler *FLC*-Sf2 resulted in a reduction in methylation at CG sites within repetitive DNA sequences. This is consistent with published results (Vongs *et al.*, 1993; Finnegan *et al.*, 1996; Ronemus *et al.*, 1996). Demethylation caused by both the AMT construct and the *ddm1* mutation promoted flowering in a genetic background with equivalent *FRI* and *FLC* alleles, compared to C24 and Col backcrossed lines. This result stands in contrast to the delayed flowering of the *ddm1* mutant in the Col background (Kakutani, 1997).

The promotion of flowering by demethylation in C24 is correlated with a reduction in the expression of the floral repressor *FLC* (Sheldon *et al.*, 1999). Analysis of the AMT and *ddm1* backcrossed lines showed that the promotion of flowering also correlated with a reduction in *FLC* expression and the concomitant upregulation of the floral promoter *SOC1* in both lines. Therefore, *FLC* is downregulated in a *ddm1* mutant background, as well as by AMT-induced demethylation. The effect of a *ddm1* mutation on *FLC* expression has not been tested before, as the *ddm1* mutation arose in Columbia, an ecotype in which *FLC* expression can not readily be detected by Northern analysis (Sheldon *et al.*, 1999).

Repetitive DNA sequences are demethylated in early generations of *ddm1*, but single-copy sequences are not (this chapter; Vongs *et al.*, 1993; Kakutani *et al.*, 1996). The methylated state of the *FWA* gene in the *ddm1* BC5⊗ line is a measure of the effect of the *ddm1* mutation on single-copy methylation in the second generation of homozygosity of the mutation. Demethylation and up-regulation of *FWA* occurs only in later generations of the *ddm1* mutant, in which single-copy sequence methylation of several genes is decreased

(Kakutani *et al.*, 1996; Soppe *et al.*, 2000). As *FWA* is not demethylated in the *ddm1* backcrossed line, other single-copy sequences are also unlikely to be demethylated. These observations suggest that the down-regulation of *FLC* expression in low methylation backgrounds is not accomplished by direct single-copy sequence demethylation of *FLC* or of a repressor of *FLC*. This is also consistent with the observation that there is no change in the methylation status of *FLC* in vernalised plants, in which *FLC* expression is decreased, compared to the methylation status of *FLC* in unvernalised plants (EJ Finnegan, personal communication).

Because DDM1 is apparently a chromatin remodelling protein (Jeddeloh *et al.*, 1999; Brzeski and Jerzmanowski, 2003), these results suggest that the demethylation-induced down-regulation of *FLC* is indirect and that the expression of *FLC* is likely to be regulated by a change in chromatin structure. This is also supportive of current models of the molecular mechanism of vernalisation, which show that after the expression of *FLC* is initially decreased, it is stably repressed by *VRN2,* a member of the Polycomb Group proteins that establish a repressive state via effects on higher order chromatin structure (Gendall *et al.*, 2001; Levy *et al.*, 2002).

A reduction in the expression of *UFC*, a gene closely linked to *FLC,* in both the AMT and *ddm1* backcrossed lines also suggests that *FLC* repression is likely to be part of a more widespread effect of methylation or chromatin remodelling over a larger area of the genome rather than just a gene-specific effect. Neither *FLC* or *UFC* regulate each other transcriptionally; *UFC* expression is unaffected in an *flc* null mutant line, and over-expression of *UFC* does not alter the expression of *FLC* (EJ Finnegan, personal communication). Therefore, the simultaneous down-regulation of *UFC* and *FLC* can not be explained by a simple case of one gene affecting the expression of another. Further

support for the repression of *FLC* being part of a widespread effect comes from the observation that in the *flc-11* mutant, in which two copies of T-DNA containing the *NptII* gene are inserted between *UFC* and *FLC,* the expression of *UFC, FLC* and the two *NptII* transgenes is down-regulated by vernalisation and in plants with low levels of DNA methylation (EJ Finnegan, personal communication).

Although *FLC* expression was reduced in both the AMT and *ddm1* backcrossed lines, flowering of the Ler *FLC*-Col/AMT line was delayed in comparison to both the Ler *FLC*-Sf2/*ddm1* line and the Ler *FLC*-Col parental line, which has moderate levels of *FLC* expression (Sheldon *et al.*, 1999). The reduction in *FLC* expression is associated with very early flowering of the Ler *FLC*-Sf2/*ddm1* line, but an equivalent level of *FLC* expression is associated with later flowering of the Ler *FLC*-Col/AMT line. The delay in flowering in the AMT backcrossed line compared to the *ddm1* backcrossed line correlated with the demethylation and expression of the floral repressor *FWA*. The expression of *FWA* in the *fwa* mutant delays flowering (Koornneef *et al.*, 1991; Soppe *et al.*, 2000). *FWA*, which acts downstream of *FLC* (Figure 1.2), is thought to indirectly repress flowering downstream of the floral promoter *FT* (Onouchi et al., 2000). Presumably, the expression of *FWA* in the Ler *FLC*-Col/AMT line partially counteracts the lower level of *FLC* expression, thereby delaying flowering.

The opposing effects of demethylation in the C24 and Ler ecotypes have recently been traced to differential effects on the expression of *FLC*, which was down-regulated in C24, and *FWA*, which was upregulated in Ler (Genger *et al.*, 2003). In C24, expression of AMT did cause demethylation and expression of *FWA*, but this did not delay flowering. The C24 *FWA* allele has two mutations compared with the Ler allele, one of which is in a conserved

region; it may be non-functional, or a downstream step in the *FWA* pathway may be lacking in C24 (Genger *et al.*, 2003).

The work reported in this chapter has shown that both AMT and *ddm1* can promote flowering in a background with high *FLC* levels, by down-regulating *FLC* expression. In addition, *FWA* is demethylated and up-regulated in AMT backcrossed plants but not in the *ddm1* backcrossed plants, because its expression is determined by single copy sequence demethylation, and *ddm1* only affects repeated sequences in early generations. Therefore, although the exact mechanism of *FLC* down-regulation in response to demethylation is not yet clear, it appears likely to occur via an effect on chromatin structure, probably involving repeat sequence demethylation, rather than as a result of single-copy sequence demethylation.

Chapter 5: Interactions between GA, vernalisation and demethylation in the promotion of flowering

5.1 Introduction

The initiation of flowering requires a plant to respond to and integrate many exogenous and endogenous signals, resulting in the activation of flowering genes and subsequent up-regulation of floral meristem identity genes. In the *Arabidopsis* C24 ecotype, two important signals that promote flowering are gibberellins (GAs) and vernalisation. GAs are a large family of diterpene compounds (Phillips, 1998) which influence myriad aspects of plant growth, including germination, stem elongation, floral induction, seed development and fruit development (Pharis and King, 1985; Davies, 1995). GA is likely to promote flowering via up-regulation of the floral promoter *SOC1* (Onouchi *et al.*, 2000; Samach *et al.*, 2000) and the floral meristem identity gene *LFY* (Blazquez *et al.*, 1998). Vernalisation, a prolonged exposure to cold, induces flowering in many late flowering ecotypes of *Arabidopsis* (Napp-Zinn, 1985). The ability of different ecotypes to respond to vernalisation is conferred by the *FRI* and *FLC* genes; the MADS-box transcription factor FLC represses flowering, and FRI acts to up-regulate *FLC*. Vernalisation promotes flowering by down-regulating *FLC* expression (Michaels and Amasino, 1999a; Sheldon *et al.*, 1999).

Whilst the four major floral promotive pathways can each independently promote flowering (Figure 1.2), many points of interaction exist between them (Figure 1.3). Years of

investigation have produced inconclusive evidence of interactions between the GA and vernalisation pathways, with some groups suggesting that they share a common pathway, and others declaring that they operate by completely separate pathways. For instance, plants that flower early in response to vernalisation also flower early in response to GA, suggestive of a common pathway (Pharis and King, 1985), but flowering is accelerated by GA to the same extent in both vernalisation-responsive and vernalisation non-responsive mutants, indicative of separate pathways (Chandler and Dean, 1994). Further support for a common pathway was suggested by observations of an increase in GA content following vernalisation of radish plants (Suge, 1970) and a decrease in the levels of kaurenoic acid, a GA precursor compound, in vernalised *Thlaspi arvense* plants, indicating increased GA turnover (Hazebroek and Metzger, 1990). The alteration of GA metabolism in *Thlaspi* occurred only in shoot tips, which are the site of perception of the vernalisation signal (Wellensiek, 1964), and not in leaves, strongly suggesting that the increase in flux through the GA biosynthesis pathway is in some way connected with vernalisation (Hazebroek and Metzger, 1990). The expression of three genes encoding GA biosynthesis enzymes in *Eustoma grandiflorum* has recently been shown to be regulated by vernalisation (Mino *et al.*, 2003).

The behaviour of some GA mutants has also pointed towards interactions between GA and vernalisation. The highly GA-deficient *ga1-3* mutant (Koornneef *et al.*, 1983; Sun and Kamiya, 1994) flowers early in response to exogenous GA in short (8-hour) days, but not in response to vernalisation (Wilson *et al.*, 1992), indicating that plants require a minimum level of GA for vernalisation to be effective. However, *ga1-3*, which does not flower at all in either 8 hour or 10 hour photoperiods, has since been shown to respond to vernalisation in 10 hour days, flowering after 35 days (Michaels and Amasino, 1999b), arguing against vernalisation acting through the GA pathway. Similarly, the late flowering GA non-

responsive *gai* mutant (Koornneef *et al.*, 1985) responds to vernalisation in SD to the same extent as wild type plants, with a 36% promotion of flowering in response to vernalisation (Wilson *et al.*, 1992).

The *ga1-3* and *gai* mutants were isolated in the genetic background of Landsberg *erecta* (Ler), which has very low *FLC* expression levels (Michaels and Amasino, 1999a; Sheldon *et al.*, 1999). As *FLC* is a key regulator of the vernalisation response, analysis of interactions between GA and vernalisation may be more revealing if done in the context of moderate to high levels of *FLC* expression. Links between *FLC* and GA have been suggested by several observations. Exogenous GA does not affect *FLC* expression levels, indicating that the promotive effect of GA is downstream of *FLC* (Sheldon *et al.*, 1999). A mutant over-expressing *FLC* requires repeated applications of GA to flower, suggesting that the *FLC* product might lower the effectiveness of applied GA (Sheldon *et al.*, 1999) and loss of FLC function gives rise to phenotypes reminiscent of an enhancement of the GA response, such as early germination and elongated hypocotyls (D Bagnall, cited in Dennis *et al.*, 2000), suggesting that in wild type plants, FLC may repress GA responses.

Like vernalisation and GA, a loss of DNA methylation also promotes flowering in C24 (Finnegan *et al.*, 1998). The role of demethylation in the promotion of flowering is not yet completely clear and evidence exists for its involvement in more than one capacity. Like vernalisation, demethylation promotes flowering via a reduction of *FLC* expression (Sheldon *et al.*, 1999), indicating that demethylation acts partly via the vernalisation pathway. However, investigations of the GA-responsiveness of antisense methyltransferase (AMT) suggested that reduced methylation could also partially substitute for the promotive effect of GA (Genger, 2000). GA promoted flowering of AMT

plants to a lesser extent than it did of C24, and GA reduced the vernalisation responsiveness of C24, but not of AMT plants (Genger, 2000).

As demethylation is able to partly substitute for both vernalisation and a GA treatment, this could occur as steps within the same pathway, or demethylation could promote flowering by more than one pathway. Preliminary experiments in this laboratory attempted to investigate this by analysing progeny of a cross between AMT and GA-insensitive *gai* mutant plants (Genger, 2000). The *gai* mutant has a gain-of-function mutation in *GAI*, a negative regulator of the GA signal transduction pathway; a 17 amino acid deletion in the amino-terminal domain of the mutant gai protein causes a structural change, leading to constitutive repression of GA responses (Peng *et al.*, 1997). Progeny of AMT x *gai* plants were late flowering in comparison to AMT plants with an unimpaired GA perception pathway, suggesting that the promotive effect of demethylation partly depended on activation of the GA pathway (Genger, 2000). However, this preliminary investigation lacked a wild type control line and did not incorporate analysis of the effect of exogenous GA or vernalisation, or attempt to determine the underlying molecular mechanisms to explain the observations. The experiments reported in this chapter were designed to further analyse the possible interactions between GA, vernalisation and demethylation-induced promotion of flowering in a background with elevated *FLC* expression levels.

5.2 Materials and Methods

5.2.1. Production of F1 lines

Crosses made use of the late flowering *gai* mutant (Koornneef *et al.*, 1985) in the Landsberg *erecta* (Ler) background and C24 plants homozygous for an antisense against

the *METI* methyltransferase (anti-methyltransferase, AMT) which has approximately 10 % of wild type levels of CG methylation (Finnegan *et al.*, 1996). Crosses were made between the two wild types, Ler and C24 (designated *GAI/GAI*); *gai* and C24 (designated *gai/GAI*); Ler and AMT (designated *GAI/GAI*-AMT); and *gai* and AMT (designated *gai/GAI*-AMT). F1 seed generated from these crosses gave rise to plants that were heterozygous at *gai/GAI* or homozygous *GAI/GAI* wild type, with either normal or low levels of methylation. All F1 plants were heterozygous for the *FRI*-late allele from C24.

5.2.2 Flowering time experiments

Seeds were treated as per section 2.9 and grown in SD conditions (8 h light, 16 h dark) in individual test tubes. The light intensity ranged from 130–170 μE across the cabinet and over the course of the experiment. Racks containing tubes were moved daily to ensure that plants received equal illumination. Flowering time was measured as rosette leaf number (RLN) and total leaf number (TLN). RLN and TLN was recorded after flowering had occurred, or for those plants that did not flower, at the termination of the experiment when the media had completely dehydrated. In these cases, the RLN and TLN recorded is an underestimation of the true leaf number at flowering.

5.2.3. PCR screening and DNA methylation analysis

After flowering or at the termination of the flowering experiment, a single leaf from each plant was removed for PCR analysis of *nga III* SSLP markers specific to Ler and C24 as per section 4.2.3, to verify that only heterozygotes were scored for flowering time (results not shown). The *GAI/GAI*-AMT and *gai/GAI*-AMT plants were also screened for the presence of the *METI* antisense transgene by PCR as per section 2.4 (results not shown).

The methylation levels of DNA extracted from pooled leaf samples of plants were analysed using methylation-sensitive restriction enzymes as per section 2.6.1.

5.2.4. *FLC* and *SOC1* expression analysis

F1 seed generated from the four crosses described above and seed from the four parental lines (C24, AMT, Ler and *gai*) were grown for 24 days on 140mm diameter Petri plates in the same light regime as described (section 5.2.1). Single leaves from individual F1 plants were screened using *nga III* SSLP markers as per section 4.2.3 to identify heterozygotes (results not shown) and leaves from heterozygous plants were pooled. Total RNA was extracted from parental lines and pooled F1 samples as per section 2.7. RNA was separated on agarose gels and hybridized to *FLC* and *SOC1* riboprobes as per section 2.8.1.

5.3 Results

5.3.1 The *gai* mutation blocks the early flowering response to GA

Preliminary investigations that compared the flowering times of progeny of *gai x* AMT plants with progeny of Ler x AMT plants, in which the GA-signalling pathway was not impaired, suggested that demethylation promoted flowering partly by a GA-dependent pathway (Genger, 2000). However, as the response of the *gai* heterozygotes to exogenous GA was not tested, this conclusion is speculative. In the Ler background, *gai/GAI* heterozygotes are intermediate to *GAI/GAI* and *gai/gai* homozygotes for both response to endogenous GA (measured as plant height) and sensitivity to exogenous GA (Koornneef *et al.*, 1985). If the *gai* x AMT heterozygotes, which are in the Ler x C24

background, proved to be responsive to GA, then the conclusion that the demethylation-induced promotion of flowering was dependent on GA is not valid.

The flowering time of the *gai/GAI* heterozygotes in the Ler x C24 background was compared to that of homozygous *GAI/GAI* plants in the same background. *GAI/GAI* plants flowered with 33.5 rosette leaves (RL), whereas *gai/GAI* plants flowered with 40.1 RL (Table 5.1). A proportion of plants in both lines (52 % of *GAI/GAI* plants and 24 % of *gai/GAI* plants) failed to bolt before the media had dehydrated. Therefore, although leaf counting was continued until senescence occurred, these RLNs are an underestimation of the true leaf number at flowering. However, using these data as they stand, flowering appears to be delayed in the *gai/GAI* heterozygotes compared to the *GAI/GAI* homozygotes, suggesting that the response to endogenous GA is impaired in these plants, and that the response to GA is a component of flowering.

When the response to exogenous GA was tested, the results substantiated the suggestion that the response to GA was impaired in *gai/GAI* plants. The effect of exogenous GA was determined using TLN as a measurement of flowering time, because elongation of the internodes between rosette leaves in GA-treated plants made the distinction between rosette and cauline leaves unclear. Exogenous GA promoted flowering of *GAI/GAI* plants, which flowered with 29.1 total leaves (TL) compared to 36.6 TL of untreated plants, 52 % of which failed to flower. The early flowering response of the *GAI/GAI* plants to GA was mirrored in their morphology; whereas untreated plants had a clearly formed rosette, elongation of the internodes between rosette leaves in the GA-treated plants caused bolting to occur before rosette leaf production had ceased (Figure 5.1A). When *gai/GAI* plants were treated with exogenous GA, they flowered with 54.1 TL, compared to 45.7 TL of untreated *gai/GAI* plants (Table 5.1). As mentioned, 24 % of the untreated *gai/GAI*

Treatment	*GAI/GAI*	*gai/GAI*	*GAI/GAI-AMT*	*gai/GAI-AMT*
Control (RLN)	33.5 ± 1.9 [A, B] n = 17 (9)	40.1 ± 2.9 [B,C] n = 21 (5)	16.3 ± 2.9 [A,D] n = 7	24.9 ± 4.3 [C,D] n = 8
+ vernalisation (RLN)	12.7 ± 0.4 [E,F] n = 17	14.3 ± 0.6 [E,G] n = 16	8.9 ± 0.5 [F,H] n = 12	11.1 ± 0.6 [G,H] n = 9
Control (TLN)	36.6 ± 1.6 [I] n = 17 (9)	45.7 ± 3.2 [J] n = 21 (5)	22.5 ± 2.4 [K] n = 7	28.6 ± 3.2 [L] n = 8
+ GA (TLN)	29.1 ± 1.4 [I] n = 17	54.1 ± 1.6 [J] n = 19 (1)	17.5 ± 1.4 [K] n = 6	28.7 ± 2.7 [L] n = 7

Table 5.1 Flowering time, expressed either as rosette leaf number (RLN) or total leaf number (TLN) of *GAI/GAI* homozygotes and *gai/GAI* heterozygotes with or without AMT (*METI* antisense construct), in the presence or absence of a vernalisation or GA treatment. Average number of leaves ± standard error is shown for each treatment. n = number of plants. Figures in brackets denote number of plants that had not bolted by the termination of the experiment. The average leaf number in those lines is therefore an underestimation of the true leaf number.

[A,C,F,G,I] significantly different; $p<0.01$

[B,D,H,J] significantly different; $p<0.05$

[E,K,L] not significantly different; $p>0.05$

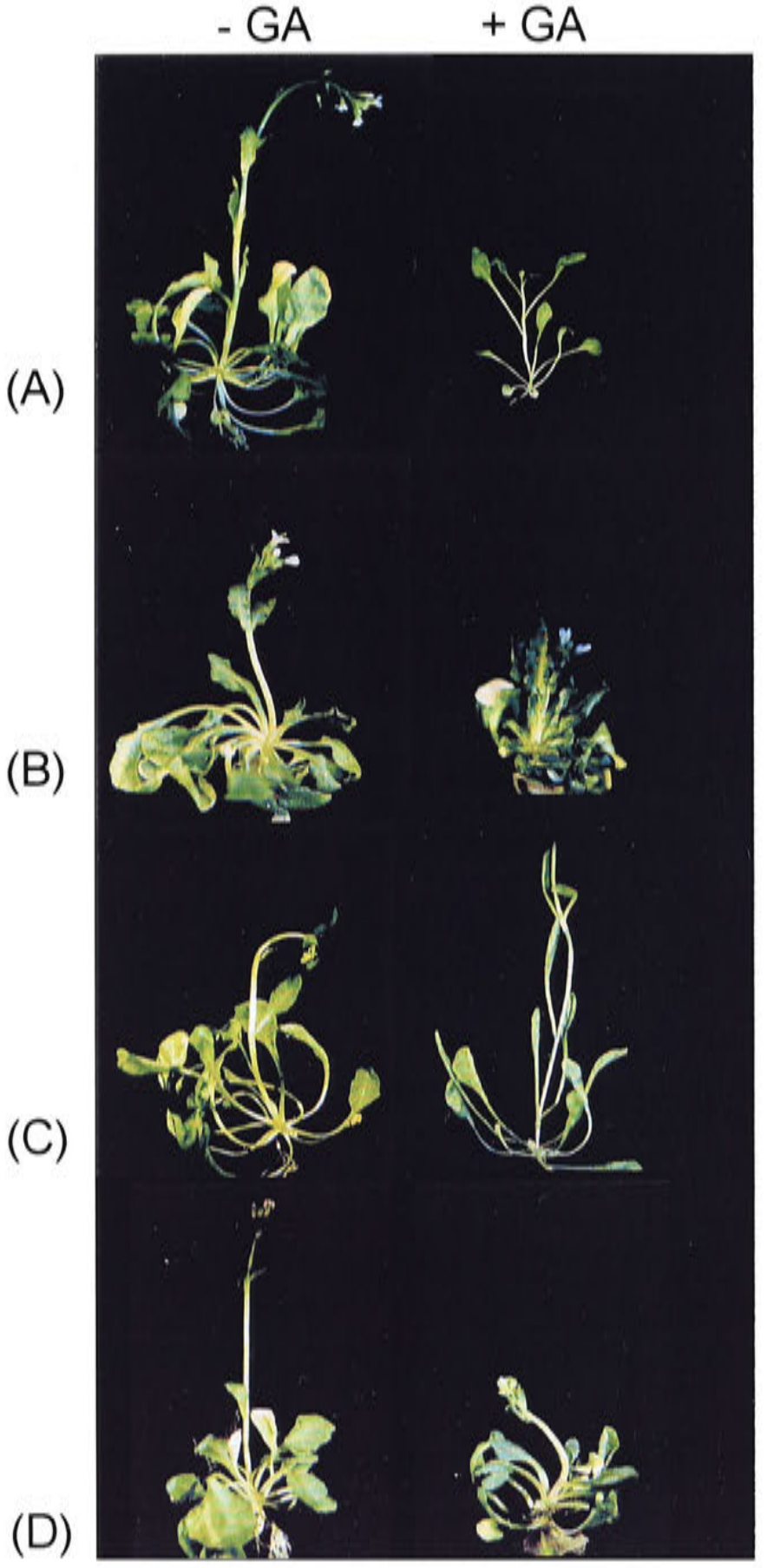


Figure 5.1 Representative F1 plants of each cross grown in the presence and absence of GA. Photographs were taken within three days of the first visible flower opening, hence plants are not necessarily of the same age. **(A)**, *GAI/GAI*. **(B)**, *gai/GAI*. **(C)**, *GAI/GAI*-AMT. **(D)**, *gai/GAI*-AMT.

plants did not flower within the time of the experiment due to media dehydration, so 45.7 TL is an underestimation of their true TLN, whereas only one of the GA-treated *gai/GAI* plants did not flower, so that 54.1 TL is a fair estimation of their TLN. These data suggest that flowering is not promoted by exogenous GA in the *gai/GAI* heterozygotes. This conclusion is supported by the observation that GA-treated *GAI/GAI* plants flowered much earlier than GA-treated *gai/GAI* plants (29.1 TL vs 54.1 TL, Table 5.1). The delay in flowering of *gai/GAI* plants in the presence of exogenous GA also supports the trend towards later flowering seen in untreated *gai/GAI* plants compared to untreated *GAI/GAI* plants. *gai/GAI* plants were generally shorter than the *GAI/GAI* plants (Figure 5.1A vs B), consistent with the *gai* mutation causing a dwarf phenotype (Koornneef *et al.*, 1985), which was retained in the presence of GA (Figure 5.1B).

Together, the above results suggest that the early flowering response to endogenous and exogenous GA is blocked in *gai/GAI* heterozygotes in the Ler x C24 background, and that consequently, like other aspects of GA signalling, the GA pathway to flowering is impaired in these plants. The results also imply that the GA pathway to flowering is operational under these conditions in the wild type plants. Therefore, together with the wild type *GAI/GAI* plants, the *gai/GAI* plants offered an ideal opportunity to study the effects of vernalisation and demethylation on flowering time and *FLC* expression in the presence and absence of a functional GA pathway.

5.3.2 The *gai* mutation partially blocks the early flowering response to demethylation

In the C24 ecotype, flowering is promoted by GA, vernalisation and demethylation (Finnegan *et al.*, 1998; Genger, 2000). Demethylation and vernalisation promote flowering

via the down-regulation of *FLC* expression (Sheldon *et al.*, 1999), and it has been suggested that demethylation might also promote flowering via an interaction with GA (Genger, 2000). To investigate whether demethylation would still promote flowering in the absence of a functional GA pathway, Ler and *gai* plants were crossed to C24 plants containing the AMT construct which had 10 % of wild type CG methylation levels (Finnegan *et al.*, 1996). DNA extracted from C24, *gai/GAI, GAI/GAI*-AMT and *gai/GAI*-AMT plants was analysed using the methylation-sensitive enzymes *Msp*I and *Hpa*II, which detect a loss of methylation within CCGG sites. There was a clear reduction in methylation in DNA of the *GAI/GAI*-AMT and *gai/GAI*-AMT plants compared to wild type C24 and *gai/GAI* DNA, as indicated by the degree of hybridisation to the digested fraction of DNA in the AMT lines (Figure 5.2; compare lanes 5 and 6 to lanes 2 and 4).

To determine whether demethylation promotes flowering to the same extent in GA-responsive and GA non-responsive backgrounds, the flowering time of *GAI/GAI*-AMT plants was compared to *GAI/GAI* control plants with normal methylation levels. *GAI/GAI*-AMT plants flowered with 16.3 RL, compared to 33.5 RL of *GAI/GAI* control plants, equivalent to at least a 51 % promotion of flowering (Table 5.1). The flowering time of the *gai/GAI*-AMT line was then compared to *gai/GAI* plants with normal methylation levels, to observe the effect of demethylation in the absence of a functional GA pathway. *gai/GAI* plants flowered with 40.1 RL, whereas *gai/GAI*-AMT plants flowered significantly earlier, with 24.9 RL, equivalent to a 38 % promotion of flowering (Table 5.1). Therefore, demethylation appears to promote flowering slightly more in the presence of a functional GA pathway than in the absence of a functional GA pathway. These observations suggest that the promotion of flowering by demethylation might be partially affected by a plant's ability to perceive or respond to GA.

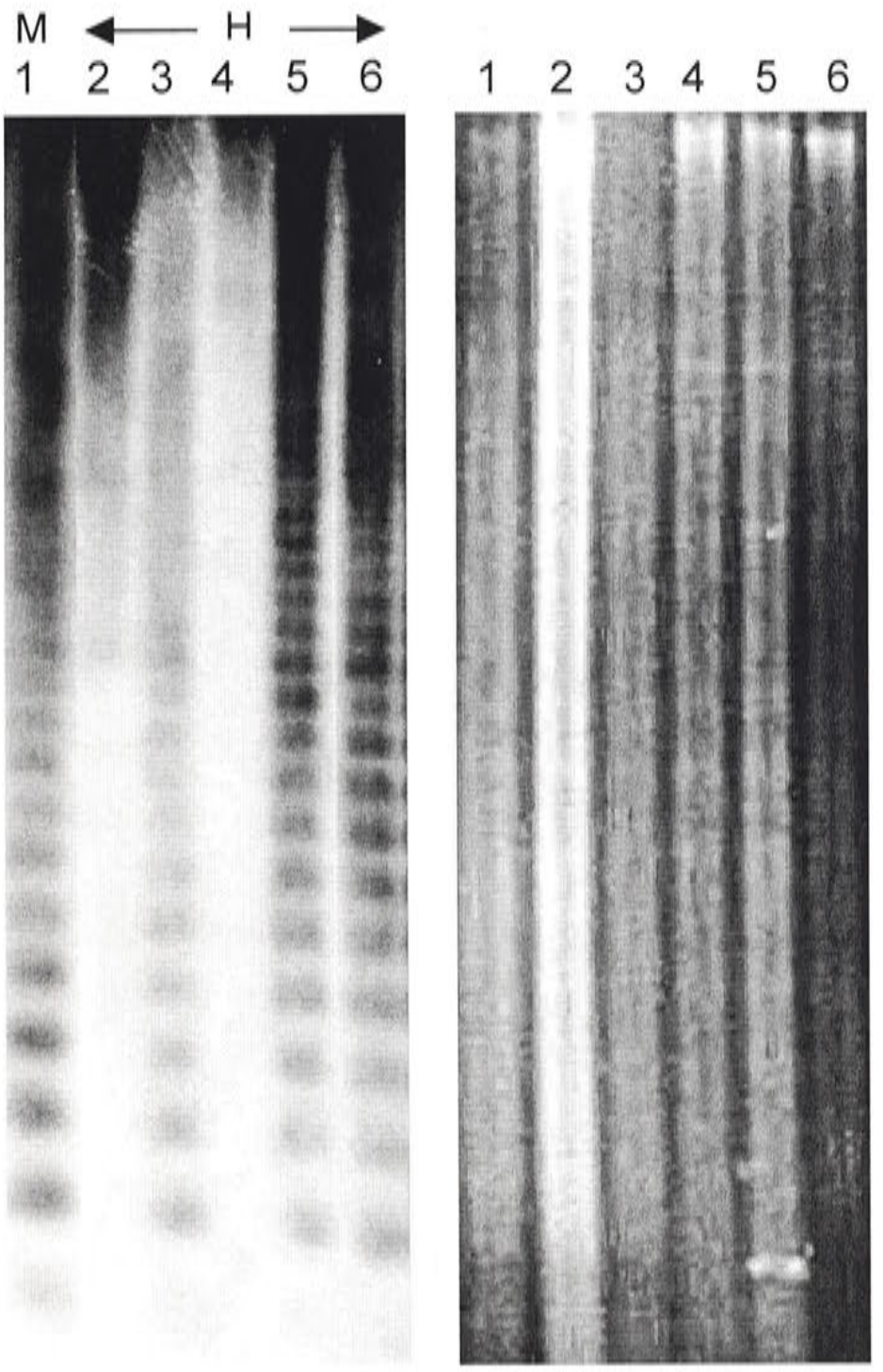


Figure 5.2 Analysis of methylation at CG sites in DNA from C24, *METI* antisense (AMT) and F1 plants with normal and low levels of methylation. DNA was digested with MspI (M) or HpaII (H) and hybridized to a 180 bp centromeric repeat probe. The EtBr stained gel is shown on the right. Lane 1 & 2, C24; lane 3, AMT; lane 4, *gai/GAI*; lane 5, *gai/GAI*-AMT; lane 6, *GAI/GAI*-AMT.

To determine whether a loss of DNA methylation altered the early flowering response to GA in wild type plants, the flowering time of *GAI/GAI*-AMT plants was measured in the presence and absence of exogenous GA. The GA-induced promotion of flowering in *GAI/GAI*-AMT plants is reduced compared to wild type *GAI/GAI* plants (Table 5.1); therefore, demethylation partially substitutes for GA, though not necessarily by the same mechanism. GA-treated *GAI/GAI*-AMT plants flowered with 17.5 TL, compared to 22.5 TL of untreated *GAI/GAI*-AMT plants (Table 5.1). Although this is not a statistically significant promotion of flowering, probably due to the small number of plants being compared, some response to GA is evident in the elongated hypocotyl phenotype of the GA-treated *GAI/GAI*-AMT plants compared to control *GAI/GAI*-AMT plants (Figure 5.1C).

The lack of a GA response in *gai/GAI* plants was reflected in the behaviour of the *gai/GAI*-AMT plants. GA-treated *gai/GAI*-AMT plants flowered with 28.7 TL compared to 28.6 TL of untreated *gai/GAI*-AMT plants (Table 5.1). This lack of a GA response is supported by the absence of an elongated internode phenotype in the GA-treated *gai/GAI*-AMT plants (Figure 5.1D).

As mentioned, demethylation in the *gai/GAI* background resulted in plants that flowered with 24.9 RL; however, demethylation in the *GAI/GAI* background resulted in plants that flowered with 16.3 RL, significantly earlier than the *gai/GAI*-AMT plants. Therefore, although flowering is promoted by demethylation in the absence of a functional GA pathway, it appears to be promoted still further in plants that possess a functional GA pathway. This result could suggest that the demethylation-induced promotion of flowering in *GAI/GAI*-AMT plants is partially dependent on GA; alternatively, perhaps *gai* delays flowering in the *gai/GAI*-AMT plants compared to *GAI/GAI*-AMT plants via a completely separate pathway that counteracts the effects of demethylation.

5.3.3 The *gai* mutation does not block the response to vernalisation

It has been proposed that GA operates downstream of *FLC*, a key regulator of the vernalisation response (Sheldon *et al.*, 1999). Previous investigations of the role of GA in the vernalisation response (e.g. Wilson *et al.*, 1992) were carried out in the Ler background, where levels of *FLC* expression are inherently low (Michaels and Amasino, 1999a; Sheldon *et al.*, 1999). To investigate interactions between GA and vernalisation further, the response of *gai/GAI* plants to vernalisation was tested in a background which has moderately high *FLC* expression levels, being a cross between Ler and C24, an ecotype with very high levels of *FLC* expression (Sheldon *et al.*, 1999; see also section 5.3.4). If GA and vernalisation do operate within the same pathway, the *gai* mutation should affect the ability of the plants to respond to vernalisation. The flowering time of *GAI/GAI* plants was compared to that of *gai/GAI* plants with and without a three-week vernalisation treatment. *GAI/GAI* plants flowered with 33.5 RL, but after vernalisation, they flowered with 12.7 RL (Table 5.1). The *gai/GAI* plants responded similarly, flowering with 14.3 RL after a vernalisation treatment, compared to 40.1 RL of unvernalised *gai/GAI* plants (Table 5.1).

These results demonstrate that in a background where *FLC* levels are moderately high, plants that are unable to respond to GA still respond to vernalisation. Consistent with this, the flowering time of vernalised *GAI/GAI* and vernalised *gai/GAI* plants is not significantly different (Table 5.1), indicating that the *gai* mutation has no effect on the promotion of flowering by the vernalisation pathway, and supporting the notion of GA and vernalisation acting through separate pathways. This contrasts with the effect of the *gai* mutation on the demethylation-induced promotion of flowering described in section 5.3.2.

To determine whether vernalisation affects the apparent interactions between demethylation and GA, the flowering time of vernalised *GAI/GAI*-AMT and *gai/GAI*-AMT plants was measured. Vernalised *GAI/GAI*-AMT plants flowered with 8.9 RL, significantly earlier than vernalised *GAI/GAI* plants, which flowered with 12.7 RL (Table 5.1), indicating that the effects of vernalisation and demethylation are partially additive. This is consistent with results seen for vernalised C24 and vernalised AMT plants (Finnegan *et al.*, 1998), showing that vernalisation does not saturate the early flowering response, as demethylation promotes flowering still further in vernalised plants. This additive behaviour was also observed in the plants with a non-functional GA pathway, where vernalised *gai/GAI*-AMT plants flowered with 11.1 RL, significantly earlier than vernalised *gai/GAI* plants which flowered with 14.3 RL (Table 5.1).

The observation that demethylation and vernalisation are additive in both the presence and absence of *gai* provides additional support for the premise that GA and vernalisation act within separate pathways. However, vernalised *GAI/GAI-AMT* plants flower significantly earlier than vernalised *gai/GAI-AMT* plants (8.9 RL vs 11.1 RL, Table 5.1). This observation is consistent with the earlier suggestion of a GA-dependent aspect of demethylation-induced flowering, and suggests that vernalisation cannot compensate for the apparent interaction between GA and demethylation.

5.3.4 Analysis of *FLC* and *SOC1* expression

5.3.4.1 FLC and SOC1 expression in parental lines

The results of the previous sections suggested that GA and vernalisation promote flowering by separate pathways, but that demethylation, which promotes flowering partly

via the GA-independent vernalisation pathway, might also promote flowering by a GA-dependent pathway. To investigate the molecular mechanisms underlying these observations, the expression of the floral repressor *FLC* and the floral promoter *SOC1* was analysed. *FLC* expression is reduced by both vernalisation and demethylation (Sheldon *et al.*, 1999). *SOC1* is a direct target of repression by *FLC* (Hepworth *et al.*, 2002) and is up-regulated by vernalisation and demethylation, due to relief of repression by *FLC* (Lee *et al.*, 2000). *SOC1* is also up-regulated by GA (Borner *et al.*, 2000), by the photoperiod pathway via *CONSTANS* (Samach *et al.*, 2000; Hepworth *et al.*, 2002), and is developmentally regulated, with its expression increasing as plants approach flowering (Lee *et al.*, 2000).

The levels of *FLC* and *SOC1* expression were initially analysed in C24, AMT, Ler and *gai*, the four parental lines used to generate the F1 lines.

In C24, *FLC* is expressed at a high level (Figure 5.3A, left panel), which represses *SOC1* expression (Figure 5.3A, right panel). In AMT plants with low methylation levels, *FLC* expression is reduced and the expression of *SOC1* increases. Ler has low *FLC* levels, partly due to the nature of its *FRI* allele (Sheldon *et al.*, 1999), and has a very high level of *SOC1* expression (Figure 5.3A, right panel). The *gai* mutant in the Ler background also has low *FLC* expression levels (Figure 5.3A, left panel). The levels of *FLC* expression correlate with flowering times of the C24 and AMT lines, where AMT is early flowering compared to C24 (Finnegan *et al.*, 1998), but the late flowering of *gai* compared to Ler (Koornneef *et al.*, 1985) is not associated with increased *FLC* expression. *SOC1* expression is lower in *gai* than in Ler (Figure 5.3A, right panel), consistent both with GA-induced up-regulation of *SOC1* being affected in *gai* plants, and with the later flowering of *gai* plants causing a delay in the developmental up-regulation of *SOC1*.

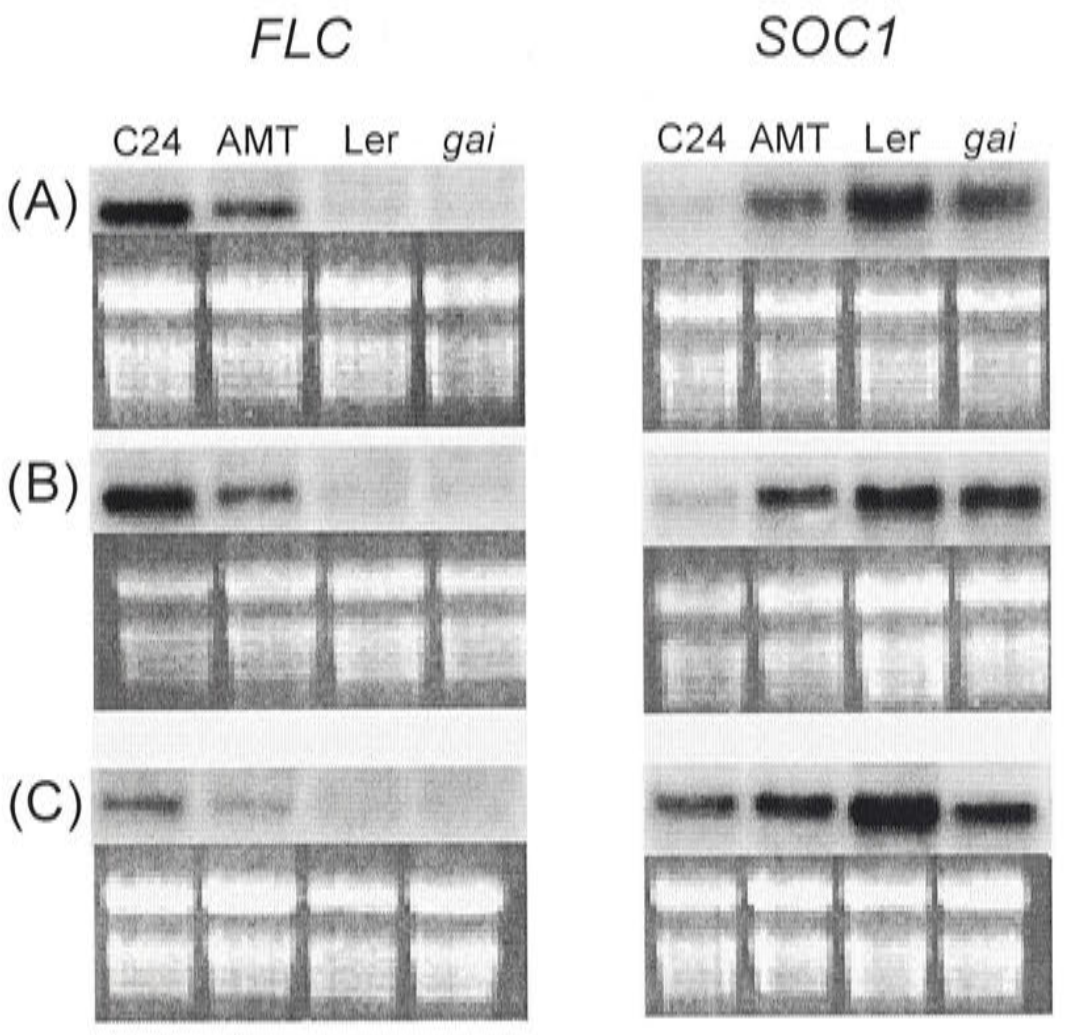


Figure 5.3 Level of *FLC* and *SOC1* expression in parental lines in the presence and absence of vernalisation or GA treatment. (A), control; (B), + GA; (C), + vernalisation. The EtBr stained gels are shown below for loading comparisons. All samples probed with *FLC* were run on the same gel, and hence are directly comparable; the same applies to all samples probed with *SOC1*.

When the four parental lines were treated with GA (Figure 5.3B), the expression of *FLC* was unchanged, consistent with reports that *FLC* acts upstream of GA (Sheldon *et al.*, 1999). The level of *SOC1* expression increased slightly in GA-treated C24, AMT and Ler lines, again consistent with up-regulation of *SOC1* by GA (Borner *et al.*, 2000). Surprisingly, the level of *SOC1* also appeared to increase slightly in the GA-treated *gai* plants; however, there is relatively more RNA in the GA-treated sample (Figure 5.3, compare far right lanes of panel A and B), which is likely to account for this.

Vernalisation decreased the level of *FLC* expression in C24 and AMT plants, and resulted in up-regulation of *SOC1* (Figure 5.3C). The low level of *FLC* expression in vernalised Ler and *gai* plants was comparable to that in the unvernalised controls, as expected, and *SOC1* was also upregulated (Figure 5.3C). The level of *SOC1* expression in vernalised Ler plants, with a functional GA pathway, was much higher than in vernalised *gai* plants (Figure 5.3C). This could be due to inhibition of GA-mediated up-regulation of *SOC1* expression (Borner *et al.*, 2000) in *gai*. However, as several Ler plants had begun to bolt before harvesting, they were more advanced developmentally, which could also account for the increase in *SOC1* expression in Ler compared to *gai*.

Taken together, the analysis of *FLC* and *SOC1* expression in parental lines is consistent with GA and vernalisation promoting flowering via separate pathways; whereas vernalisation decreases *FLC* and increases *SOC1*, exogenous GA does not significantly affect their expression. However, the lack of a functional GA pathway appears to affect *SOC1* expression, indicating that the vernalisation and GA pathways integrate at *SOC1*.

5.3.4.2 FLC and SOC1 expression in F1 lines

In the F1 lines, the level of *FLC* expression in *GAI/GAI* and *gai/GAI* lines is intermediate between C24 and Ler (Figure 5.4A compared to Figure 5.3A; note that in Figure 5.4A, the *GAI/GAI* sample is substantially underloaded in comparison to the other samples).

The level of *FLC* expression was reduced in the *GAI/GAI*-AMT and *gai/GAI*-AMT plants (Figure 5.4A), correlating with the earlier flowering of these lines compared to the *GAI/GAI* and *gai/GAI* lines (Table 5.1). *SOC1* expression increased in both AMT lines compared to the control lines (Figure 5.4A), consistent with the promotion of flowering in these lines. As *SOC1* expression increased to the same extent in both the *GAI/GAI*-AMT and *gai/GAI*-AMT lines, there does not appear to be a direct role for GA in the up-regulation of *SOC1* in response to demethylation.

In the GA-treated plants, *FLC* expression appeared to increase beyond that of untreated plants (Figure 5.4A vs B). This is inconsistent both with GA acting downstream of *FLC* (Sheldon *et al.*, 1999) and with the GA-induced promotion of flowering in the *GAI/GAI* and *GAI/GAI*-AMT lines seen in the previous flowering experiment (Table 5.1). However, as the flowering time was not measured on the same batch of plants as those used for expression analysis, it is not known if flowering was promoted in the GA-treated plants used for RNA isolation. Because of this, the apparent increase in *FLC* expression is assumed to be an artefact, as it was not observed in parental lines treated with GA (Figure 5.3A vs B). However, consistent with the increase in *FLC* expression in the GA-treated F1 lines, *SOC1* expression decreased in these lines (Figure 5.4B). The repression of *SOC1* by *FLC* therefore appears to override the expected up-regulation of *SOC1* expression by GA (Borner *et al.*, 2000).

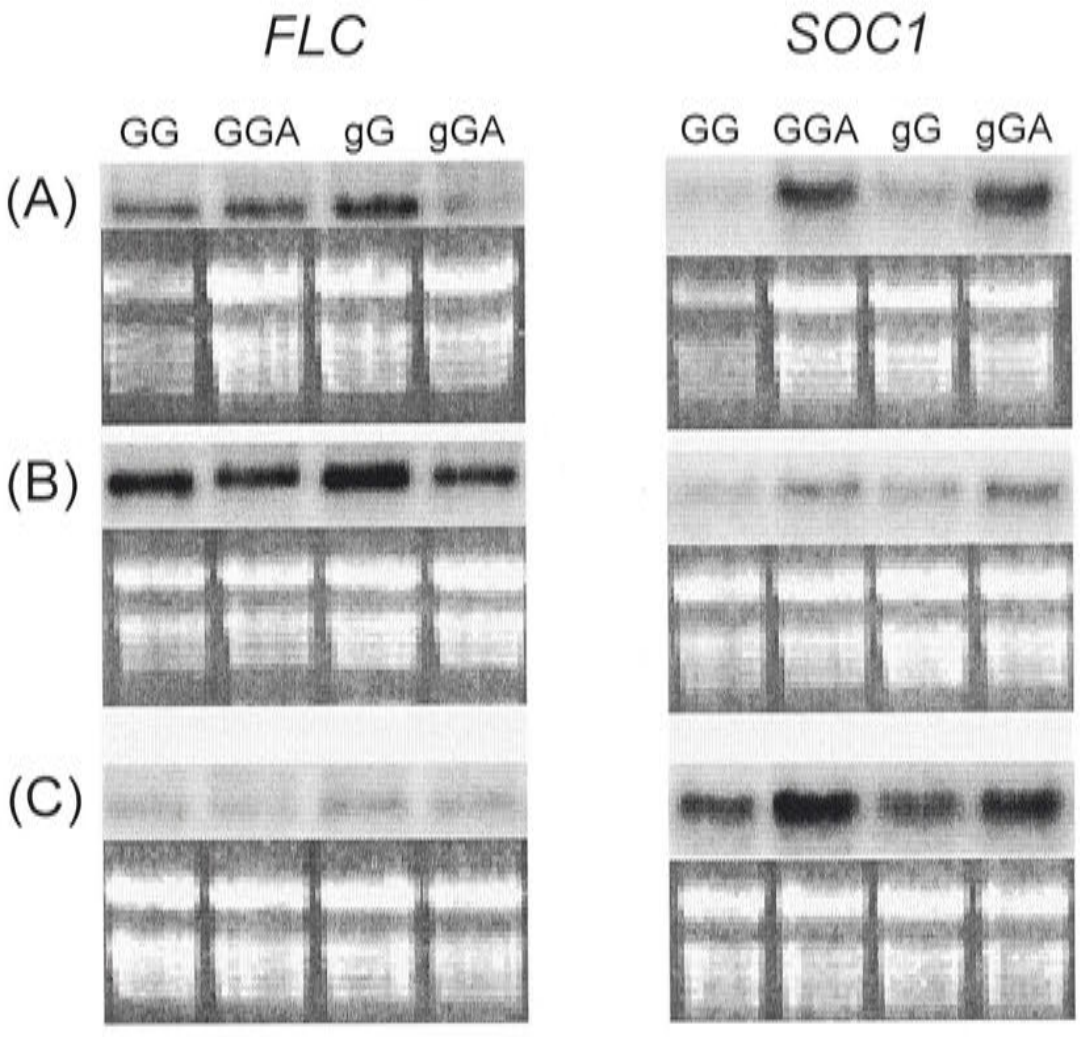


Figure 5.4 Level of *FLC* and *SOC1* expression in F1 lines in the presence and absence of vernalisation or GA treatment. (A), control; (B), + GA; (C), + vernalisation. GG = *GAI/GAI* (Ler *x* C24); GGA= *GAI/GAI*-AMT (Ler *x* AMT); gG = *gai/GAI* (*gai x* C24); gGA = *gai/GAI*-AMT (*gai x* AMT). The EtBr stained gels are shown below for loading comparisons. All samples probed with *FLC* were run on the same gel, and hence are directly comparable; the same applies to all samples probed with *SOC1*. Note that in (A), the *GAI/GAI* sample is relatively underloaded in both gels.

Vernalisation resulted in a reduction in *FLC* expression in all four F1 lines (Figure 5.4A vs C), consistent with vernalisation being independent of the *gai* mutation. The decrease in *FLC* expression correlated with the concomitant up-regulation of *SOC1* (Figure 5.4C) and is consistent with the promotion of flowering of vernalised plants observed in the flowering time experiment (Table 5.1). Vernalised *GAI/GAI*-AMT and *gai/GAI*-AMT plants had higher levels of *SOC1* expression than the vernalised *GAI/GAI* or *gai/GAI* plants with normal methylation levels, even though all lines appeared to have comparable, low levels of *FLC* expression (Figure 5.4C); however, it is possible that the *FLC* levels do vary, but below the limit of detection. The increase in *SOC1* expression in the vernalised AMT lines is consistent with their earlier flowering compared to the vernalised *GAI/GAI* and *gai/GAI* lines seen in the previous flowering experiment (Table 5.1). Although none of the F1 plants used for RNA isolation had bolted before being harvested, the increase in *SOC1* expression in vernalised AMT plants could be due to developmental upregulation.

Interestingly, the expression of *SOC1* appeared to be slightly higher in vernalised *GAI/GAI-AMT* plants than vernalised *gai/GAI-AMT* plants (Figure 5.4C). This is consistent with the earlier flowering of vernalised *GAI/GAI-AMT* plants compared to vernalised *gai/GAI-AMT* plants (Table 5.1). In contrast, the flowering time of vernalised *GAI/GAI* and *gai/GAI* plants was not significantly different (Table 5.1), and *SOC1* expression was more comparable in these lines (Figure 5.4C).

The flowering time experiments suggested that demethylation promoted flowering in *gai/GAI*-AMT plants in comparison to *gai/GAI* plants via a GA-independent pathway. This correlated with decreased *FLC* expression in *gai/GAI*-AMT plants compared to *gai/GAI* plants. However, flowering was promoted even further in *GAI/GAI*-AMT plants with a functional GA pathway, and this was not correlated with a further decrease in *FLC* expression; if anything, *FLC* expression is higher in *GAI/GAI-AMT* than *gai/GAI-AMT*

plants (Figure 5.4A). It is therefore possible that the earlier flowering of *GAI/GAI-AMT* compared to *gai/GAI-AMT* may occur via an *FLC*-independent pathway, possibly involving GA. Taken together, these results indicate that demethylation might promote flowering by more than one mechanism.

5.4 Discussion

Flowering in *Arabidopsis* can be promoted by many signals, including GA, vernalisation, and demethylation. The generation of heterozygous *gai* plants enabled an investigation of interactions between GA, vernalisation and demethylation in plants with elevated levels of *FLC* expression.

Although the *gai/GAI* heterozygous plants used in the experiments reported here were unable to respond to GA, they flowered early after a vernalisation treatment. The vernalisation-induced promotion of flowering correlated with a decrease in *FLC* expression levels and increase in *SOC1* expression levels. The response to vernalisation, but not to GA, is consistent with vernalisation and GA promoting flowering via separate pathways. Demethylation promoted flowering in *gai/GAI*-AMT plants compared to *gai/GAI* plants, indicating that a GA-independent aspect of demethylation-induced promotion of flowering exists; this correlated with a decrease in *FLC* expression and relief of *FLC*-mediated *SOC1* repression, and is consistent with GA acting downstream of *FLC* (Sheldon *et al.*, 1999).

Demethylation appeared to promote flowering to a greater extent in GA-responsive plants than in GA non-responsive plants, suggesting the down-regulation of *FLC,* although the major influence of demethylation on flowering, may not be the only one, and that part of the demethylation-induced early flowering response could be dependent on a functional

GA pathway. The GA-dependent demethylation-induced promotion of flowering in *GAI/GAI*-AMT plants was not associated with any further decrease in *FLC* expression, consistent with GA acting downstream of *FLC* (Sheldon *et al.*, 1999). The down-regulation of *SOC1* expression in *gai* plants compared to wild type Ler is consistent with GA up-regulating *SOC1* (Borner *et al.*, 2000), and could suggest that *gai* acts, at least in part, upstream of *SOC1* (Figure 5.5). However, the GA-dependent aspect of demethylation-induced flowering in *GAI/GAI-AMT* plants was not associated with an obvious increase in *SOC1* expression. Perhaps the effect of demethylation is downstream of *SOC1*, for example, at *LEAFY* (Figure 5.5).

Vernalised *GAI/GAI* and *gai/GAI* lines, which flowered at the same time, had moderately high *FLC* levels before vernalisation. As *FLC* is the key regulator of the vernalisation pathway, the vernalisation-induced decrease in *FLC* expression is likely to be responsible for these similar flowering times. In contrast, in the already reduced *FLC* background of the AMT F1 lines, vernalised *GAI/GAI-AMT* plants flowered earlier than vernalised *gai/GAI-AMT* plants, which correlated with an increase in *SOC1* expression but no detectable change in *FLC* expression. This is consistent with the recent discovery of a minor *FLC*-independent vernalisation pathway (Michaels *et al.*, 2003), a pathway intimated several years ago by the observation that Ler and *gai* plants show a vernalisation response in SD (Wilson *et al.*, 1992) despite their low *FLC* levels (Sheldon *et al.*, 1999; this chapter). In line with this, *SOC1* expression is up-regulated by vernalisation in an *flc* null mutant (Moon *et al.*, 2003). The up-regulation of *SOC1* in the *flc* mutant occurs only when the GA pathway is also active (Moon *et al.*, 2003).

Because vernalised *GAI/GAI-AMT* plants flower earlier than vernalised *gai/GAI-AMT* plants, this pathway might also involve GA (Figure 5.5). The *FLC*-independent

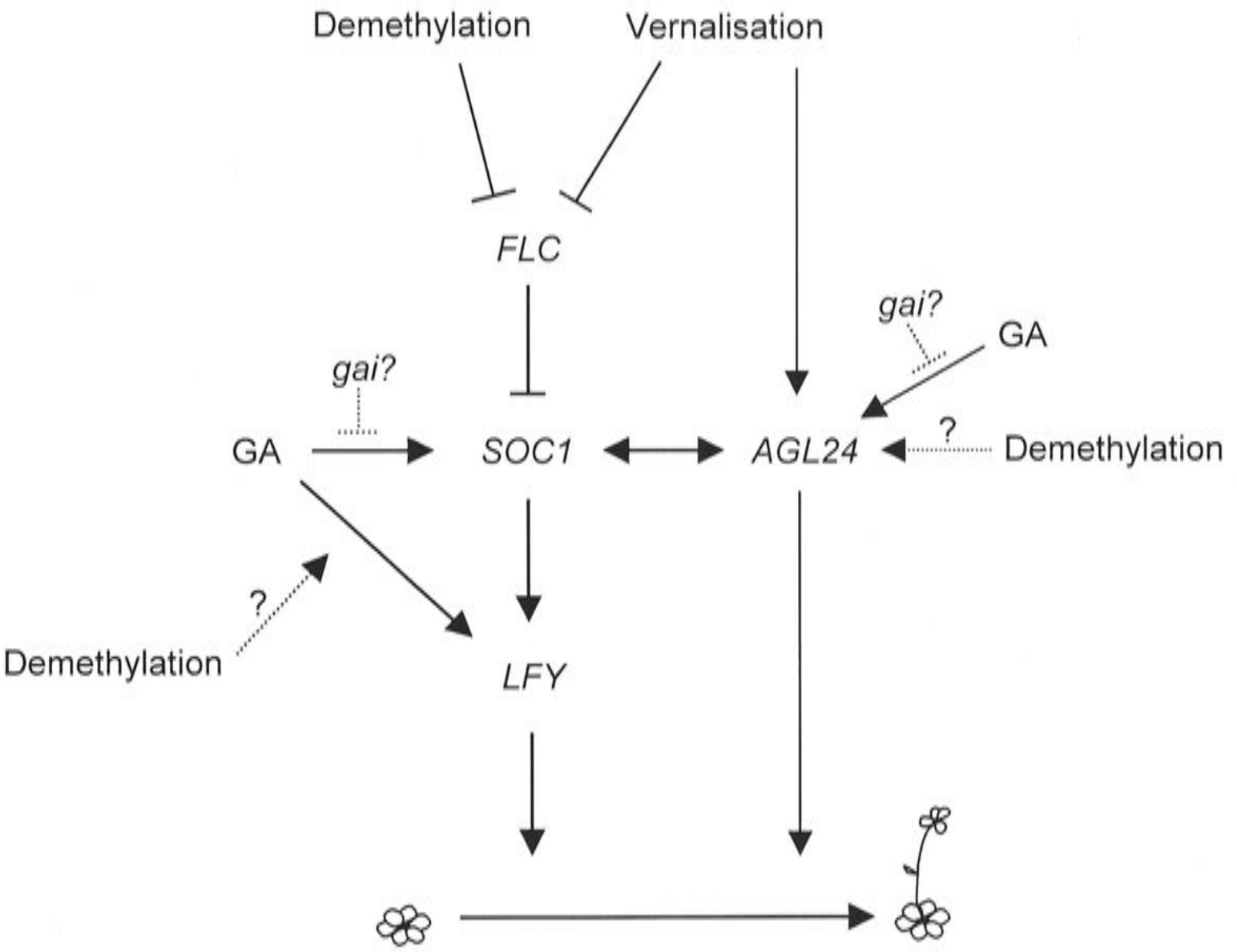


Figure 5.5. Interactions between GA, vernalisation and demethylation in the promotion of flowering, incorporating potential sites of action of demethylation and *gai*.

vernalisation pathway involves up-regulation of the MADS-box transcription factor *AGL24*, which reciprocally up-regulates *SOC1* (Michaels *et al.*, 2003; Figure 5.5). *AGL24* is also up-regulated by GA (Yu et al., 2002). However, the GA-dependent up-regulation of *AGL24* is markedly reduced in a *soc1* mutant (Yu *et al.*, 2002), so perhaps the up-regulation of *AGL24* by GA is mediated via *SOC1* (Figure 5.5). *SOC1* expression increases in *GAI/GAI*-AMT and *gai/GAI*-AMT plants compared to plants with normal levels of methylation, which might be independent of *FLC* (this chapter). Perhaps *AGL24* expression is also regulated by methylation (Figure 5.5); if so, it could be a likely candidate to explain the promotion of flowering in vernalised and unvernalised *GAI/GAI*-AMT plants compared to *gai/GAI*-AMT plants.

Together, the results of this chapter suggest that vernalisation and GA promote flowering by separate pathways which integrate at *SOC1*, and that the early flowering response to demethylation is mostly due to a GA-independent down-regulation of *FLC* expression. However, the data also suggest that there may be other aspects to demethylation-induced early flowering, which could possibly occur via an *FLC*-independent and/or a GA-dependent mechanism.

Chapter 6: General Discussion

6.1 Roles of DNA methylation in promoting flowering

A loss of DNA methylation disrupts many aspects of plant development. In *Arabidopsis*, demethylation results in a range of phenotypic abnormalities such as decreased fertility, altered leaf dimensions, reduced apical dominance and alteration of flowering time (Finnegan *et al.*, 1996; Ronemus *et al.*, 1996). Introduction of an antisense construct against the predominant DNA methyltransferase, *METI,* reduces methylation of CG dinucleotides to 10 % of wild type levels. This observation, combined with the fact that particular sequences become hypermethylated in *METI* antisense plants (Kishimoto *et al.*, 2001), suggests other DNA methyltransferases may have roles in plant development. Apart from *METI,* there are 9 DNA methyltransferase genes in *Arabidopsis* (The Arabidopsis Genome Initiative, 2000), any of which could carry out methylation. The role of *METII*, a methyltransferase belonging to the same class as *METI*, was investigated in this thesis.

The introduction of *METII* silencing constructs into the Arabidopsis C24 ecotype resulted in plants containing as little as 20 % of wild type levels of *METII* expression. *METII* transgenic plants displayed no apparent change in morphology and had no detectable decrease in either overall methylation levels, or in methylation of either CG or CTG in repeated sequences, compared to wild type plants. A similar lack of morphological change has been reported in two other DNA methyltransferase mutants, *cmt3* in *Arabidopsis* (Lindroth *et al.*, 2001) and *dnmt2* in mice (Okano *et al.*, 1998a). Despite the lack of a detectable decrease in methylation levels, *METII* transgenic plants with at least a 60 %

reduction in *METII* expression levels flowered significantly earlier than wild type plants. Early flowering of *METI* antisense plants in the C24 ecotype is correlated with a reduction in expression of the floral repressor *FLC* (Sheldon *et al.*, 1999). In contrast, the early flowering of *METII* transgenic plants was not associated with any apparent reduction in *FLC* expression, suggesting that METII has different target sequences to METI. The lack of detectable demethylation in *METII* transgenic plants suggests that these targets may only be a small proportion of the genome. Highly selective, sequence-specific methylation has been observed in human DNA (Xu *et al.*, 1999); perhaps METII methylates specific sequences that are important in the floral transition. The effect on the floral transition could be indirect, as comparative microarray analysis of a line with low levels of *METII* expression and wild type plants showed that the early flowering of the *METII* line was associated with upregulation of a number of photosynthetic genes. This suggests that METII might be involved in the regulation of either photosynthetic genes or an upstream regulator of these genes. The up-regulation of chloroplast-encoded genes in the *METII* transgenic line could be due to effects on a nuclear-encoded upstream regulator of these genes, as METII is predicted to be a nuclear-localised protein.

Demethylation resulting from introduction of a *METI* antisense construct (AMT) promotes flowering in the C24 ecotype, due to down-regulation of the floral repressor *FLC* (Sheldon *et al.*, 1999). In contrast, in the Columbia (Col) ecotype, where *FLC* expression is very low, demethylation resulting from introduction of an AMT construct or from a mutation in the *DDM1* gene delays flowering (Ronemus *et al.*, 1996; Kakutani *et al.*, 1996; Kakutani, 1997). Flowering is affected immediately in Col-AMT plants, whereas late flowering in *ddm1* mutants is only observed after several generations of inbreeding. The late flowering phenotype of AMT in Col and *ddm1* is associated with demethylation and expression of the normally silent *FWA* gene, which encodes a floral repressor (Soppe *et al.*, 2000). Late

flowering does not occur in C24-AMT plants; the *FWA* allele of C24 may be non-functional (Genger *et al.*, 2003).

Because the Col ecotype, in which the *ddm1* mutation was isolated, has low levels of *FLC* expression (Sheldon *et al.*, 1999), it was unknown whether like AMT, a mutation in the *DDM1* gene could down-regulate *FLC* expression. To investigate this possibility, AMT and *ddm1* were backcrossed to Landsberg *erecta* lines containing dominant expressed alleles of *FLC* and methylated, silenced *FWA* alleles. CG methylation within repetitive DNA sequences was reduced and *FLC* expression was down-regulated in both the AMT and *ddm1* backgrounds, promoting flowering. In contrast to the *ddm1* mutant in the Col background, in which flowering time was affected only after six generations of inbreeding (Kakutani, 1997), a promotion of flowering was seen in the second generation of homozygosity of *ddm1* in the experiments reported here.

Unlike *FLC*, which was repressed in both AMT and *ddm1* backgrounds, the single-copy gene *FWA* was demethylated, and hence expressed, only in the AMT background. This suggests that down-regulation of *FLC* is not controlled by single-copy sequence methylation. A reduction in the expression of *UFC*, a gene adjacent to *FLC*, in both the AMT and *ddm1* backcrossed lines further suggested that *FLC* repression is likely to be part of a widespread effect of DNA methylation or chromatin remodelling over a larger area of the genome. DDM1 is homologous to chromatin remodelling proteins (Jeddeloh *et al.*, 1999; Brzeski and Jerzmanowski, 2003); loss of *DDM1* activity results in loss of DNA methylation (Vongs *et al.*, 1993) and redistribution of histone H3 lysine 9 (H3K9) methylation (Soppe *et al.*, 2002). Repression of *FLC* by an effect on chromatin structure is consistent with the need for the *VRN2* gene for *FLC* repression (Gendall *et al.*, 2001). VRN2 is a Polycomb group protein that in other organisms has been shown to establish a

repressive state via effects on higher order chromatin structure (Gendall *et al.*, 2001; Birve *et al.*, 2001).

Like demethylation (Finnegan *et al.*, 1998), vernalisation promotes flowering by down-regulating *FLC* expression (Sheldon *et al.*, 1999). Vernalisation has been suggested to both interact with and to be independent of the GA pathway, though the evidence supporting these suggestions is inconclusive and studies have previously only been done in low *FLC* backgrounds. The experiments reported here used plants with elevated levels of *FLC* expression. Plants in which GA signalling was impaired by mutation of *gai* showed an equivalent response to vernalisation as that seen in *GAI* wild type plants, supporting the suggestion that the promotion of flowering by vernalisation is independent of GA. Crossing the *gai* mutant to AMT plants with low methylation levels showed that demethylation promotes flowering via a GA-independent pathway which relies on down-regulation of *FLC*. However, demethylation promoted flowering still further in a GA-responsive background, which suggested that demethylation might also interact with the GA pathway to flowering. Expression of the floral integrator *SOC1*, known to be the target of FLC repression and GA activation (Borner *et* al., 2000; Onouchi *et al.*, 2000; Samach *et al.*, 2000) was up-regulated in vernalised *GAI/GAI*-AMT and *gai/GAI*-AMT plants compared to plants with normal levels of methylation, but *FLC* levels were comparable in all vernalised genotypes tested. *SOC1* expression was up-regulated slightly more in vernalised *GAI/GAI*-AMT than *gai/GAI*-AMT plants, suggesting that a recently identified *FLC*-independent vernalisation pathway (Michaels *et al.*, 2003) could be active in *GAI/GAI*-AMT plants, and that this may involve GA.

6.2 Future directions

6.2.1 Further characterisation of the role of METII

Silencing *METII* promotes flowering and correlates with upregulation of photosynthetic genes. To ascertain whether METII has a role in the regulation of photosynthetic genes, the microarray analysis should be repeated with line 7.2, as well the other two *METII* lines. If photosynthetic genes are up-regulated in the early flowering line 6.2, but are not up-regulated in line 13.1 which has no change in flowering time, then it could be concluded that the early flowering phenotype was definitely correlated with the changes in photosynthetic gene expression, and that *METII* has a role in the regulation of these genes. Alternatively, if photosynthetic genes are not up-regulated in the second early flowering line, then it is possible that insertion of the *METII* construct in line 7.2 has disrupted the expression of a gene that regulates photosynthesis. To investigate this, the insertion site of the *METII* construct could be mapped using TAIL PCR and the role of any gene disrupted by the insertion could be determined.

To further determine the role *METII* plays in flowering, *Arabidopsis* could be transformed with hairpin constructs directed against the genes identified as up-regulated in the microarray analysis. Such lines could be used to investigate whether these genes affect flowering time; a delay in flowering in these lines in comparison to the overexpressing lines would be predicted. Genomic sequencing of bisulphite-treated DNA of the genes with altered expression would reveal any specific sites of methylation by METII, although it is possible that transcriptional regulators of these genes may be targets of METII. Further characterisation of the cDNA clones and proteins of unknown function that are up- and down-regulated in the *METII* transgenic line may reveal their functions and help to identify other potential targets of METII. In addition, as the slides used in the analysis described

here contained only half of the *Arabidopsis* genes, microarray analysis could be performed on the remainder of the *Arabidopsis* genome.

The role of *METII* in the vernalisation response is unclear. Although a reduction in *METII* expression promotes flowering, it has no detectable effect on *FLC* expression, unlike in *METI* antisense plants. Analysis of antisense *METI x METII* lines is currently underway (EJ Finnegan, personal communication). It will be interesting to see if compromising the expression of both genes can completely substitute for a vernalisation treatment. Several lines of evidence suggest that cooperative behaviour exists between methyltransferases, so it is possible that METI and METII could also cooperate. For example, in a human cell line, disrupting *DNMT1* caused a 20 % decrease in methylation and disrupting *DNMT3b* caused only a 3 % decrease, but double knockout lines had a 95 % reduction in methylation levels (Rhee *et al.*, 2002). In *Drosophila,* co-expression of *DNMT1* and *DNMT3a* resulted in an increase in methylation levels beyond that seen in singly expressing lines (Lyko *et al.*, 1999); this cooperative behaviour might result from DNMT1 maintaining the methylation installed by the *de novo* DNMT3a/3b enzymes.

Further characterisation of METII function could also be undertaken. *In vitro* assays of protein function using baculovirus-mediated expression could help to determine the target sequences of METII. Later generations of *METII* transgenic lines could be examined to determine if the early flowering phenotype is stable, or if other phenotypes arise after repeated selfing. Any subtle phenotypes might be revealed by a more in-depth expression analysis than has been done to this point (Genger, 2000). Promoter-GUS or GFP fusion constructs could be utilised to analyse *METII* expression in different stages of development and in different tissues. METI has recently been shown to maintain CG methylation during plant gametogenesis (Saze *et al.*, 2003) and Dnmt3L is expressed

during gametogenesis in mice (Bourc'his *et al.*, 2001); perhaps the influence of METII also varies within different stages of development.

Analysis of three recently available *METII* T-DNA insertion lines from the Salk collection, which include two *METIIa* (#010893 and #018896) and one *METIIb* (#048436) lines, would help to define a role for the *METII* genes. As *METII* expression is reduced to 20 % at most in the lines described here, knockout lines with zero *METII* expression could be informative. Crossing the *METIIa* and *METIIb* insertion lines may reveal functions of *METII*, especially if they are redundant. An additional T-DNA insertion line in the C24 background that flowers early and has decreased levels of *FLC* expression has insertions in three genes, one of which is in *METIIa* (C Andersson, personal communication). However, it has not yet been determined which insertion is responsible for this phenotype.

6.2.2 Further analysis of the epigenetic control of flowering

Future directions of research into the epigenetic control of flowering should include direct transformation of C24 with hairpin *METI* and *ddm1* constructs to enable a more equivalent comparison of their mechanisms of action in exactly the same genetic background. Hairpin construct technology had not been developed at the time these backcrossed lines were being generated. Comparison of the expression profiles of all *Arabidopsis* genes in the AMT and *ddm1* backcrossed lines and hairpin construct lines might reveal genes that are differentially expressed as a result of different mechanisms of demethylation and that may be involved in the down-regulation of *FLC,* as well as any other flowering time genes.

Based on the observation that single copy sequences are not demethylated in early generations of *ddm1* homozygotes (Vongs *et al.*, 1993; Kakutani *et al.*, 1995, 1996), it

seems likely that demethylation of repeat sequences appears to be related to the down-regulation of *FLC* in response to AMT or disruption of *DDM1*, whereas single-copy sequences seem to be less important. A loss of methyl cytosine caused by *ddm1* or *met1* mutations has a dramatic effect on repetitive sequences, leading to a loss of H3K9 methylation and alteration of chromatin organisation (Soppe *et al.*, 2003). Repetitive sequences are usually clustered within heterochromatic chromocentres; histone methylation is redistributed away from the chromocentres in *ddm1* and *met1* mutants (Soppe *et al.*, 2003). It is not clear why redistribution of H3K9 methylation should affect *FLC/UFC* expression. Chromatin immunoprecipitation techniques could be used to investigate whether any alteration of histone modifications, including H3K9 and H3K4 methylation or H3K9 acetylation, occurs within the *FLC/UFC* region of the AMT or *ddm1* backcrossed lines.

It is known that DDM1 is required for methylation of tandem repeats at the centromere and at other repeated sequences (Vongs *et al.*, 1993). Recombinant DDM1 can bind to and remodel DNA *in vitro* (Brzeski and Jerzmanowski, 2003). Although these authors did not observe any change in ATPase or binding activity of DDM1 in the presence or absence of methylation, this analysis was limited to the single-copy *FWA* sequence and did not include analysis of any repetitive sequences. If there are any repeated sequences near the *FLC-UFC* chromosomal region, perhaps DDM1 can recognize and bind to them, leading to remodelling of the *FLC-UFC* chromatin region.

In wild type Ler-*FLC* lines, the *FLC-UFC* region is transcriptionally active and flowering is delayed. In the *ddm1* backcrossed lines, chromatin remodelling is affected by loss of DDM1, and *FLC* expression decreases; presumably the region becomes transcriptionally inactive. In the AMT backcrossed lines, demethylation of the repeat sequences might

affect the binding of DDM1, thereby affecting chromatin remodelling, and down-regulating *FLC* expression. This could be tested by comparing the binding affinity of DDM1 for methylated and non-methylated repetitive DNA using antibodies against DDM1 in chromatin immunoprecipitation assays with chromatin from wild type and AMT plants.

In *Arabidopsis,* most repetitive DNA is found in centromeric regions. Some repetitive transposon sequences, such as the F9D12.2 Mutator-like transposon, which is located at 9 Mb on chromosome V (Figure 4.9), are methylated in wild type plants and demethylated in *ddm1* plants (Singer *et al.*, 2001). Although most repetitive transposon sequences are found in the region spanning 9-20 Mb (Figure 4.9), they also occur in the 0-8 Mb region, at a frequency of approximately 1 per 100 kb (The Arabidopsis Genome Initiative, 2000). Therefore, there could be repetitive transposon sequences around the *FLC-UFC* region, which is located at 3 Mb (Figure 4.9), although there are no obvious candidates on the BAC containing *FLC* (EJ Finnegan, personal communication). Further investigations could include analysis of the regions up and downstream of the *FLC-UFC* region, to see whether any adjacent genes are also down-regulated in the absence of DDM1 or METI function and to map the extent of the affected domain. This may provide insight into whether there is a direct role for repeated sequences in the down-regulation of *FLC* expression.

6.2.3 Further analysis of interactions between GA, demethylation and vernalisation

It is clear that further work is required to clarify the exact nature of the interactions between demethylation, vernalisation and GA. To confirm the results suggested by the experiments described in chapter 5, the flowering time experiments should be repeated using larger numbers of plants, with the same batch of plants being used for northern analysis of *FLC*

and *SOC1* expression, and *FLC/SOC1* expression should be accurately quantified. To avoid the problem of the media drying up and skewing the results of the flowering time experiment, the flowering experiment should be repeated in soil. Additionally, a flowering time experiment should be done in conjunction with repeating the expression analysis of the parental lines. Younger plants should be used for the expression analysis to avoid any effects of developmental stage on *SOC1* expression.

Future analysis of the response of the *AGL24* gene to GA and demethylation, including analysis of *AGL24* expression in AMT plants and of the methylation status of the *AGL24* gene and promoter region, may help further our understanding of the apparent interactions between GA and demethylation. Microarray analysis of the F1 lines may also reveal whether any GA-regulated genes are differentially expressed in *GAI/GAI-AMT* compared to *gai/GAI-AMT* plants. The *gai* mutant could also be directly transformed with the AMT construct and compared by microarray analysis to *gai* plants with normal methylation levels, to observe any interactions between demethylation and GA pathway in the absence of *FLC*.

6.3 Final conclusions

Flowering involves complex interactions between different external and internal stimuli and has many control points. DNA methylation is of great importance for many aspects of gene regulation in almost all species; given that demethylation has such a pleiotropic effect on plant development, it seems reasonable to assume that demethylation could affect gene expression and promote flowering via more than one pathway or mechanism. For example, METII might be involved in the regulation of some photosynthetic and other light-regulated genes, and so may be involved with the photoperiod pathway. METI has an

important role in the regulation of flowering via the vernalisation/autonomous pathways, by down-regulating *FLC*. METI may also interact with the GA pathway. Could METII or other methyltransferases have other regulatory roles, perhaps within the GA pathway, or the minor FLC-independent vernalization pathway?

Although *FLC* down-regulation is clearly the major effect of demethylation on flowering, at least by AMT (METI) induced demethylation, it might not be the only effect that demethylation has on flowering pathways. In the future, elucidation of the roles of the other methyltransferase enzymes, including METIIa, METIIb, METIII, CMT and DRM enzymes will shed more light on the complexity of the interactions between DNA methylation and plant development.

Appendix 1: Functions of known genes differentially expressed in *METII* line #7.2 and C24

Up-regulated genes:

Rubisco small subunit: a nuclear encoded gene. In response to a phytochrome signal, the rbcS gene is transcribed, by virtue of a transcriptional regulator that binds to a light-regulated element in its promoter. The SSU protein is targeted to the chloroplast where it combines with the Rubisco large subunit, which is transcribed from chloroplast DNA. Rubisco fixes both carbon and oxygen (Tobin and Silverthorne, 1985).

Light harvesting chlorophyll a/b protein: the product of the *cab* gene. Cab proteins contain antenna chlorophylls and carotenoids (Tobin and Silverthorne, 1985).

Chlorophyll a/b binding protein: pigment-binding protein. Chlorophyll reacts with oxygen to form free radicals that can damage proteins; chlorophyll is bound to pigment-binding proteins that "quench" the chlorophyll reaction, protecting the cell (Tobin and Silverthorne, 1985).

Photosystem proteins: The PSI complex transfers electrons to NADP. Reduced NADPH is used for carbon fixation. The PSII protein complex splits water and produces oxygen (Tobin and Silverthorne, 1985).

Phosphate translocator protein: a membrane-bound protein involved in exchanging orthophosphate and triose phosphate between the chloroplast and the cytosol, to enable the synthesis of sucrose (Taiz and Zeiger, 1991).

Rotamase precursor: rotamases increase the rate of protein folding by catalysing the isomerisation of bonds in oligopeptides (Fischer and Schmid, 1990)

Chalcone synthase: a light-regulated enzyme of the flavinoid/phenylpropanoid biosynthesis pathway. The products of CHS are UV-light protectants and act as a type of plant "sunscreen" (Paiva, 2000).

Vegetative storage protein: a glycoprotein with acid phosphatase activity, induced by sucrose, light and environmental stresses such as wounding and water deficiency. Synthesised and accumulated in cell vacuoles in vegetative tissue and used for growth and development of organs (Berger *et al.*, 1995; Utsugi *et al.*, 1998).

Major latex proteins: have been identified in poppy (Nessler and Burnett, 1992) and homologues have recently been found in *Arabidopsis* but no known function has been ascribed to them. The major latex protein Type 3 is an anti-microbial gene (Schenk *et al.*, 2000).

Peroxidases: are involved in the production of lignin, cross-linking of cell wall structural proteins, auxin catabolism, pathogen defence and salt tolerance (Hiraga *et al.*, 2001).

Catalase: destroys peroxide formed as a by-product of glycolate, which is the hydrolysed form of phosphoglycolate, the product of oxygen fixation by Rubisco in the process of

photorespiration (Taiz and Zeiger, 1991). *Arabidopsis* catalase genes are light-regulated (McClung, 1997).

Ubiquitin-conjugating protein (UCP): Ubiquitin binds covalently to proteins, serving as a recognition site for a large proteolytic complex. The common UCP "E2" brings ubiquitin to the site where ubiquitination occurs, and holds it there until ubiquitination has occurred (Bachmair *et al.*, 2001).

Down-regulated genes:

Nodulin-like protein: there are 20-30 different nodulins, found in legume nodules and involved in signal exchange between *Rhizobium* and nitrogen-fixing root nodules (Stougaard, 2000).

Water stress-induced protein: water deficit is a normal component of many plant developmental processes, as well as a stress induced when transpiration rate exceeds water uptake. Changes in gene expression in response to water stress can take place within minutes and a large number of genes are involved (Bray, 1997).

Amine oxidase-like protein: amine oxidase converts tryptamine into indole-3-acetaldehyde in the tryptophan-auxin metabolic pathway (Taiz and Zeiger, 1991).

TNP-2 like transposon protein: encoded by CACTA transposons. Activation of transposable elements under stress conditions is thought to provide methods of coping with varied environmental conditions by allowing for genome plasticity (He *et al.*, 2000).

Npr1-like: NPR1 encodes a cytoplasmic kinase that antagonizes a ubiquitin-mediated protein degradation pathway (Johnston *et al.*, 2001).

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